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PROTEIN METABOLISM IN RATS: ESTIMATION OF TOTAL PROTEIN TURNOVER AND CHARACTERISTICS OF IN VITRO INACTIVATION OF TYROSINE AMINOTRANSFERASE

©

BY

WAYNE THOMAS BUCKLEY

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Protein Metabolism in Rats: Estimation of Total Protein Turnover and Characteristics of In Vitro Inactivation of Tyrosine Aminotransferase" submitted by Wayne Thomas Buckley in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Animal Biochemistry.

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ABSTRACT

Two aspects of the metabolism of protein in rats were investigated. A method for estimating the rates of total protein synthesis, accretion and degradation in intact rats has been proposed and evaluated. In addition, the mechanism of intracellular protein degradation has been studied using the inactivation of tyrosine aminotransferase (L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.5) in rat liver homogenates as a model.

Quantitative parameters of phenylalanine metabolism have been used to estimate the rate of incorporation of phenylalanine into body protein. In conjunction with N balance trials as well as amino acid analyses of the feed and rat empty body mass, the rates of protein synthesis, accretion and degradation were calculated according to a simplified model of phenylalanine and protein metabolism. Rats with a mean body weight of 160 g were found to have mean rates of protein synthesis, accretion and degradation (expressed as the weight of amino acids that would be derived from the hydrolysis of the protein in question) of 1.94, 0.87 and 1.08 $g(day)^{-1}$, respectively, and rats with a mean body weight of 123 g were found to have rates of 1.56, 0.94 and 0.63 $g(day)^{-1}$, respectively. The method appears to be limited by diurnal variation in the rates of protein turnover, lack of knowledge of the specific activity of the L-[U-^{14}C]phenylalanine precursor of protein synthesis, and the difference in metabolism between L-[U-^{14}C]tyrosine produced endogenously from the hydroxylation of L-[U-^{14}C]phenylalanine and L-[U-^{14}C]tyrosine infused into the plasma. Recommendations were made to overcome several of the limitations.

The inactivation of tyrosine aminotransferase, measured in rat liver homogenates by an automated enzyme assay, was found to occur in the presence of cysteine as observed by previous workers. In addition, it was demonstrated that O_2 was required for inactivation, and that cystine could replace both O_2 and cysteine in the inactivation reaction. The particulate fraction of the homogenates was required for inactivation to proceed in the presence of cysteine, but not in the presence of cystine. The role of the particulate fraction appeared to be to catalyze the oxidation of cysteine, and not to supply cathepsins for the proteolytic inactivation of tyrosine aminotransferase as suggested by others. It was suggested that the enzyme was inactivated by the formation of a mixed disulfide with cystine, and that a reaction of this sort could be involved in the initial steps of the degradation of tyrosine aminotransferase in vivo. The results are consistent with a model of tyrosine aminotransferase degradation proposed recently in which tyrosine aminotransferase would be inactivated after formation of the apoenzyme.

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SECTION I

ESTIMATION
OF
TOTAL PROTEIN
TURNOVER

INTRODUCTION

The accretion of protein in animals is a function of both the rates of protein synthesis and protein degradation. Since the flux of amino N through body protein may be two to three times that entering the body from the diet (65), the degradation of protein, in addition to synthesis, is an important factor in determining the total mass of body protein.

The relationship between protein accretion, synthesis and degradation varies with nutritional and physiological states. All factors that affect the accretion or depletion of protein by animals must affect synthesis, degradation, or both. For example, during starvation and concomitant loss of body N, degradation must be greater than synthesis, and during growth, synthesis must be greater than degradation. The effects of many factors influencing synthesis and degradation may, however, not be predictable. For example, malnourished human infants receiving the same dietary N and having the same rate of protein accretion as recovered infants had significantly greater rates of both synthesis and degradation of body protein (49).

A number of investigations of the turnover of muscle proteins and proteins of other tissues in both domestic and laboratory animals have been reported (for examples see 4, 12, 22, 41, 48). The information obtained in these studies contributes to our knowledge of animal protein production. An understanding of nutritional and physiological factors affecting whole body protein synthesis and degradation should also be of value in the study of animal protein production. Nevertheless, attempts to estimate the rates of protein synthesis and degradation in whole animals raised for meat or other protein products do not appear to have been reported. A major reason may be the lack of a suitable method.

Although net changes in body protein may be readily estimated, the estimation of the rates of synthesis and degradation of protein has proved to be more difficult. Waterlow (64) concluded in a review published in 1969 that an adequate method for the estimation of total body protein turnover likely had not yet been developed. Section I of this thesis describes an attempt to develop a method suitable for the estimation of protein accretion, synthesis and degradation in intact animals.

LITERATURE REVIEW

Protein turnover has been defined as the flux of amino acids through protein (34). It is a term which is useful if the rate of protein synthesis equals the rate of degradation; that is, the protein pool size remains constant. Its meaning is not so clear if the size of the protein pool under consideration is changing. Nevertheless, the term, protein turnover, has been extensively used in the literature, and will be used here in reviewing the literature.

A. Methods of Estimating Protein Synthesis and Degradation.

Waterlow (64) and Neuberger and Richards (45) have reviewed methods used for the estimation of whole body protein turnover. Although it is doubtful that any of the methods reviewed have provided an accurate measurement of protein turnover (64), the results obtained have yielded useful information about protein and amino acid metabolism. Several of the methods described by Waterlow (64), and several other methods which have been reported more recently are discussed below.

In studies of protein turnover it is probably most convenient to measure protein accretion by N balance and either the rate of protein synthesis or degradation by some other method. The unmeasured component can then be determined by difference, providing a gross description of protein turnover of the animal.

Methods of estimating protein synthesis and degradation in tissues and the whole body have usually involved use of isotopes, particularly ^{15}N and ^{14}C. The advantages and disadvantages inherent with the use of isotopes have recently been appraised by Garlick and Millward (19). In their review (19), it was concluded that the disadvantages of estimating either synthesis or degradation are evenly balanced, and that the choice between the two approaches may rest on convenience or the particular goals of the experiment. In general, methods of estimating rate of synthesis are limited by a lack of knowledge about the specific activity of the precursor of protein and by diurnal variation in rate of synthesis. Methods of estimating degradation suffer from recycling of label and from effects of applying isotopes to a heterogenous mixture of protein pools.

a. Constant Infusion of [^{14}C]Lysine

Waterlow (63) and Waterlow and Stephen (66) have described a method of estimating whole body protein turnover from the flux of serum lysine in rats (66) and in men (63). The method consisted of constant intravenous infusion of L-[U-^{14}C]lysine until a plateau was established for specific activity of free lysine in the serum. From the plateau specific activity the flux of lysine through the serum was calculated. Using an estimate of the average lysine content of rat or human protein, the turnover of protein was calculated directly from the lysine flux. With control rats in the weight range of 76 to 210 g, values of 25 to 30 g(kg)$^{-1}$(day)$^{-1}$, i.e. (g protein) (kg body wt.)$^{-1}$(day)$^{-1}$, were determined in this manner (66). In human males, age 19, values of 3.1 and 3.2 g(kg)$^{-1}$(day)$^{-1}$ were measured (63).

Because of the relatively short infusion times (7 h or less), it was assumed that reentry of labelled lysine into the serum pool as a result of protein degradation could be neglected. The calculations of Aub and Waterlow (5) indicate that this assumption was valid. It was also assumed that the entire serum lysine flux was utilized for the synthesis of body protein; however, significant catabolism of serum lysine would have resulted in an overestimation of the rate of protein turnover. It was also recognized (63, 66) that the method did not account for the intracellular recycling of amino acids derived from degradation into protein synthesis. Because of recycling the specific activity of lysine at the site of protein synthesis may have been lower than serum lysine specific activity. It was estimated (66) that a correction for the expected lower specific activity would yield values for protein turnover of about 130 to 150 % of that calculated from the serum plateau specific activity.

More recently, Waterlow and Stephen (67) have estimated the turnover of muscle, liver and plasma protein of rats from the constant infusion of L-[U-^{14}C]lysine. The calculations utilized the plateau specific activity of free lysine extracted from muscle and liver tissue. Combining the values obtained for muscle, liver and plasma with estimates for skin and the remaining viscera, Waterlow and Stephen (67) estimated that the total incorporation of lysine into body protein for a 100 g rat would be 112 μmoles$(h)^{-1}$,or an equivalent of 38 g protein$(kg)^{-1}(day)^{-1}$.

Recent evidence (2) indicates that the specific activity of the amino acid precursor of protein is intermediate between the specific activity of plasma amino acids and the specific activity estimated from the extraction of intracellular amino acids. Therefore, an error resulting from the underestimation of precursor specific activity may have occurred in the later experiments of Waterlow and Stephen (67). The difference between serum and tissue specific activities indicate that the error of an overestimate of protein turnover would probably have been less than 60 %.

b. Single Injection of [^{14}C]Lysine

Using a single injection method, Arnal, Fauconneau and Pech (4) have recently estimated the rate of protein synthesis in the major tissues of the rat. Following single injections of L-[U-^{14}C]-lysine, rats were killed at intervals for up to 6 days. The changes in the intracellular specific activities of free lysine and protein-bound lysine were followed in each tissue. The rates of synthesis of protein of the blood, viscera (pooled gastrointestinal tract, liver, kidneys, spleen and pancreas), skin and muscles of the hind legs and the remaining carcass were calculated. The total rate of synthesis of protein of the tissues was estimated to be 32 $g(kg)^{-1}(day)^{-1}$ for 200 g rats. The tissues included in the estimate comprised 78 % of the body weight. Arnal, Fauconneau and Pech (4) pointed out that the most critical factor in the estimation of the rate of synthesis was the determination of the specific activity of the precursor amino acid. The possible errors in estimation of the precursor specific activity described previously for the experiments of Waterlow and Stephen (67) would also apply in this case.

c. *Constant Infusion of [^{15}N]Glycine*

Picou and Taylor-Roberts (49) have developed a technique for estimating total body protein synthesis and degradation in human infants. The technique employs the constant infusion of [^{15}N]glycine, and represents a considerable improvement over the use of single injections of [^{15}N]glycine, originally proposed by Sprinson and Rittenberg (54) and modified by San Pietro and Rittenberg (50). The theoretical model of N metabolism on which the method of Picou and Taylor-Roberts (49) is based is similar to that used in the experiments presented in this thesis (Fig. 1). The major difference is that the model of Picou and Taylor-Roberts (49) includes all the amino acids found in protein, whereas the model presented here (Fig.1) includes only phenylalanine. In the model of Picou and Taylor-Roberts (49) amino acids from the diet and from the catabolism of protein were assumed to enter a precursor pool common to protein synthesis and amino acid degradation. Because the model allows for only one protein pool and only one amino acid pool, it is very much over-simplified.

Picou and Taylor-Roberts (49) assumed that amino acids derived from the diet were metabolized in the same manner as those from the catabolism of protein. It was also assumed that [^{15}N]glycine was a valid tracer for total amino N. Experiments designed to test these assumptions indicated that, for the purposes of the experimental method, they were valid. In healthy infants the rate of protein synthesis was estimated to be about 6 g(kg)$^{-1}$(day)$^{-1}$.

Young et al. (69) have recently used a modification of the method of Picou and Taylor-Roberts (49) in a study of the effect of age on total protein synthesis in humans. It was found that the rate of protein synthesis decreased with increasing age when expressed per unit of body weight, but when expressed per unit of energy expenditure or protein consumption, protein synthesis was found to be independent of age.

Of the variety of techniques used to study protein turnover in humans (64), the recent results obtained with [^{15}N]glycine (49, 69) seem to be the most reliable, and indicate that useful information about protein turnover may be obtained from a simplified model of amino acid and protein metabolism.

B. The Use of [^{14}C]Phenylalanine in the Estimation of Protein Synthesis

In the method proposed in this thesis, an estimation of the rate of protein synthesis is based on there being a relatively limited number of catabolic and anabolic pathways of phenylalanine metabolism. Thus, it is necessary to review knowledge on the metabolism of phenylalanine in animals.

Investigations of phenylalanine metabolism have centered around the metabolic disorder known as phenylketonuria, in which the conversion of phenylalanine to tyrosine is partially or completly blocked. Grümer, Koblet and Woodard (29) have reported studies of the metabolism of [^{14}C]phenylalanine (probably L-[U-^{14}C]phenylalanine, but not specified for the experiment discussed here) in a phenylketonuric patient in whom it was estimated that only 2 % of the phenylalanine was converted to tyrosine during one turnover time of the phenylalanine pool. They found that no appreciable amount of label from [^{14}C]-phenylalanine was located in the glycine moiety of hippuric acid excreted in the urine. Since glycine can be synthesized from intermediates of gluconeogenesis, these results indicate that the metabolism of phenylalanine in this patient did not yield intermediates of glucose synthesis.

Deamination of phenylalanine, yielding cinnamic acid, is known to occur in plants (39). When [3-^{14}C]cinnamic acid was administered to rats by intraperitoneal injection it was not metabolized to $^{14}CO_2$, nor was it significantly incorporated into the tissues of the rats (56). Within 24 h, 73 % of the injected radioactivity was excreted in the urine. There is a possibility, in studies of this sort, that a labelled compound which was injected may not be metabolized in the same manner as the same compound produced endogenously. Nevertheless, these results suggest that if significant deamination of phenylalanine occurs in mammals, the product is not further metabolized, but is excreted in the urine.

In mammals, the transamination of phenylalanine yields phenylpyruvic acid (39). The product of the decarboxylation of phenylpyruvic acid, phenylacetic acid, is excreted in the urine in a conjugated form. In most animals it is excreted as phenylacetylglycine, while in man and the chimpanzee it is excreted as phenylacetylglutamine (39). Phenylacetylglutamine is a normal constituent of human urine (55). When phenylacetic acid was administered orally to humans, it was virtually all recovered in the urine as phenylacetylglutamine (68), indicating that, except for conjugation with glutamine, phenylacetic acid is metabolically inert in humans.

Wadman et al. (62) found that urinary excretion of phenylalanine, benzoic acid (free and conjugated), phenylacetic acid (free and conjugated), mandelic acid, *o*-hydroxyphenylacetic acid, phenyllactic acid and phenylpyruvic acid in three typical cases of phenylketonuria accounted for 80 to 90 % of the dietary intake of phenylalanine. The ages of the patients were 1, 2 and 19 years. Since there is probably some residual phenylalanine 4-monooxygenase (L-phenylalanine, tetrahydropteridine:oxygen oxidoreductase [4-hydroxylating], EC 1.14.16.1) activity in the phenylketonuric condition (60) and some phenylalanine must have been required for the accretion of protein, the results of Wadman et al. (62) indicate that the metabolism of phenylalanine, other than protein synthesis and hydroxylation, results in quantitative urinary excretion of the products.

Clearly phenylalanine may be used in protein synthesis or may be converted to other small molecules, but on the basis of the reports discussed here it has been assumed that the products of the latter conversions of phenylalanine, other than tyrosine, are excreted in the urine.

MATERIALS AND METHODS

A. *Animals*

Male, Sprague-Dawley rats were raised in individual metabolism cages, with local lighting from 6 AM to 6 PM. All rats were from the colony maintained by the Department of Animal Science, the University of Alberta. An automatic feeding device (Appendix A) was constructed in order to impose a continuous feeding regimen on the rats. The automatic feeder delivered an average of 0.27 g of a standard laboratory rat diet to each rat at intervals of 30 min. Under these conditions the rats grew an average of 3.0 $g(day)^{-1}$. Rats fed the same diet ad libitum consumed 1.5 times more daily dry matter and grew 2.0 times more rapidly.[1] The continuously fed rats were on a restricted feed intake to provide motivation to consume the feed when it was presented. Whenever the rats were observed at feeding time, the feed was consistently eaten within a few minutes. The rats were weighed every 3 or 4 days.

B. *Analytical Procedures*

Amino acid analyses of feed and rat empty body mass[2] were done by column chromatography (Amino Acid Analyzer, model JLC-5AH; Japan Electron Optics Laboratory, Co.) following hydrolysis in boiling 6 N

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[1] Four rats fed ad libitum consumed an average of 17.9 $g(day)^{-1}$ of dry matter (standard error, 0.38 $g(day)^{-1}$; number of observations, 28) and grew an average of 6.0 $g(day)^{-1}$ (standard error, 0.50 $g(day)^{-1}$; number of observations, 4).

[2] Entire body with gastrointestinal contents removed.

HCl for 24 h at atmospheric pressure.

N analyses of feed, excreta and rat empty body mass were done by the Kjeldahl method (33) using a 4 % boric acid solution to trap NH_3 during distillation.

Both N and amino acid analyses of rat empty body mass were done following lyophilization, fat extraction with petroleum ether and grinding to pass a 20 mesh screen.

Dry weight of feed was determined by drying to constant weight in a forced air oven at 107 C.

Radioisotope counting was achieved using a liquid scintillation system (Mark I Liquid Scintillation Computer, model 6860; Nuclear Chicago Corp.). Except for background measurements of expired air (see section E of Materials and Methods), samples were counted so that the theoretical standard deviation for repeat counts was less than 1 % of the total counts. Counting efficiency was determined by the channels ratio method (11).

C. N Balance

All rats were allowed to adapt to the continuous feeding regimen for at least 5 days before samples for N balance were collected. Daily feed consumption was determined as the difference between feed provided and the residue in the individual feed hoppers (see Appendix A) for each rat. Feces and urine were collected separately from each animal every day for 3 to 5 days prior to the first period of isotope administration, for 2 to 3 days between the first and second periods of isotope administration, and for 3 to 5 days after the second period of isotope administration. Urine was collected on polyethylene-backed absorbent paper (Kaydry Lab Table Soakers; Canadian Laboratory Supplies) arranged in square funnels under the metabolism cages such that the urine was absorbed while the feces rolled into flasks below the funnels. Total daily collections of feces and urine were used for N analyses. The absorbent paper, which had a N content of about 1 % of the urine collected, was digested with the urine in the Kjeldahl procedure. When the paper was collected, hair sloughed from the rat was shaken free and not included in the N analyses. There was negligible spillage of feed.

D. Administration of L-[U-^{14}C]Tyrosine and L-[U-^{14}C]Phenylalanine

a. Radiochemical Purity

L-[U-^{14}C]tyrosine, specific activity 507 mC(mmole)$^{-1}$, and L-[U-^{14}C]phenylalanine, specific activity 492 mC(mmole)$^{-1}$, both purchased from Amersham/Searle Corp. were tested for radiochemical purity using two dimensional thin layer chromatography (10). The solvents used for developing the plates were; 1) 60 % n-butanol, 20 % acetic acid and 20 % water, by weight, and 2) 75 % phenol and 25 % water. After developing the plates, the locations of the [^{14}C]amino acids were determined by autoradiography. A plate on which both [^{14}C]amino acids were applied showed that L-[U-^{14}C]tyrosine and L-[U-^{14}C]phenylalanine were effectively separated. Plates on which only one of the [^{14}C]amino acids had been applied were scraped to yield the silica gel of the origin, spot and the rest of the plate. The silica gel was then suspended in Bray's scintillation fluid (9) with the addition of Cab-O-Sil (Cabot Corp.). Of the total radioactivity on the plates, 96 and 97 % was recovered in the spots attributed to L[U-^{14}C]tyrosine and L-[U-^{14}C]phenylalanine, respectively.

b. Administration of Labelled Amino Acids

The method of estimating the rate of protein synthesis involved the administration of the [^{14}C]amino acids either with the diet (treatment A) or by constant infusion (treatment B). In treatment A the [^{14}C]amino acid was mixed with coarse feed which was then finely ground and well mixed. The specific activity of the feed was determined by extracting the isotope with 0.2 M monoethanolamine and counting in Bray's scintillation fluid (9). The specific activity varied from 4.0 to 4.5 $\mu C(g)^{-1}$ for different preparations, and the calculated specific activity was consistently within 1 % of the specific activity determined by extraction.

From 9:00 AM to 4:00 PM of the day of an isotope trial in treatment A, portions of labelled feed equal in weight and frequency to the delivery from the automatic feeder were provided to a rat held in a chamber which had an empty volume of 750 ml. The chamber consisted of a glass tube 8 cm in diameter sealed at the ends with rubber stoppers. The animal rested on a wire screen. One end was fitted with an air inlet and outlet, water supply and a stoppered tube for manual introduction of the labelled feed. During an experiment, 3 to 4 % of the feed administered was usually not consumed.

This was collected, weighed and an appropriate correction applied. The first [^{14}C]amino acid fed was L-[U-^{14}C]phenylalanine followed in 2 or 3 days by L-[U-^{14}C]tyrosine, except for 2 rats for which the order was reversed.

In treatment B, infusion was into a tail vein through a catheter which was inserted (see Appendix B for catheterization procedure) immediately before beginning the experiment. During infusion and placement of the catheter the rat was held in a glass tube of small enough diameter to restrict movement. A rubber stopper at the head end of the tube was fitted with an air inlet and outlet and a stoppered tube for introducing feed. At the rear end of the tube, the tail extended through another rubber stopper. Feces and urine were collected in a sidearm extending downward, while a wire screen encircled the interior rear 6 cm of the tube providing footing for the rat. The labelled amino acids were diluted with sterilized physiological saline and infused by means of a piston pump (Lambda Pump Series 1300, with Lambda Pump Driver, model 1301; Harvard Apparatus Co.) at a rate of about 0.3 μC(h)$^{-1}$ and in a volume of about 0.5 ml(h)$^{-1}$. The precise infusion rate was determined in each experiment by measuring volume changes in a pipette which served as a reservoir for the infusate. All equipment in contact with the infusate was either autoclaved or treated with a 0.1 % solution of alkyldimethylbenzylammonium chloride (Zephiran). The volume infused per hour corresponded approximately with the normal rate of water intake by the rats, and for this reason water was not supplied during the infusion. The infusion was usually continued for 7 h from 9:00 AM to 4:00 PM after which the catheter was removed and the rat replaced in its metabolism cage. Feeding was continued manually every 30 min throughout the infusion so that the rats continued on the feeding regimen without interruption. The feed was consistently eaten when presented. The first [^{14}C]amino acid infused was L-[U-^{14}C]phenylalanine followed in 2 or 3 days by L-[U-^{14}C]tyrosine.

E. Collection of Expired $^{14}CO_2$

Throughout the administration of [^{14}C]amino acids, expired $^{14}CO_2$ was collected (Appendix C) for 30 min of each hour. Thirty min collection periods were chosen because it was found that the quantity of expired $^{14}CO_2$ fluctuated in a 30 min cycle corresponding to the feeding schedule of the rat. Expired $^{14}CO_2$ was collected directly into a scintillation fluid (Appendix C). Using $^{14}CO_2$ released from [^{14}C]carbonate upon the addition of acid in place of the rat and the rat holding chamber, recoveries of 100±2 % of the available ^{14}C were consistently obtained with the collection system. Immediately before the beginning of each administration of [^{14}C]-amino acid to the rats, expired CO_2 was collected for a 30 min period in order to provide a correction for background activity. Prior to the first administration of [^{14}C]amino acid the background activity was negligible. Prior to the second administration of [^{14}C]amino acid the background activity was between 1 and 10 % of that collected in a 30 min sample at plateau during the administration of the second [^{14}C]amino acid.

F. *Collection of Feces and Urine During Administration of Labelled Feed*

A separate experiment with 4 typical rats was performed to study the excretion of label during the administration of [^{14}C]amino acids.

a. *Preparation of Labelled Feed*

Aliquots of the standard rat diet were labelled with L-[U-^{14}C]-phenylalanine and L-[U-^{14}C]tyrosine by preparing slurries with aqueous solutions of the labelled amino acids. After lyophilization, the resulting cakes of feed were finely ground in a Waring Blendor (Waring Products Co.), and allowed to equilibrate with the humidity in the animal room. The specific activities of the prepared feeds were determined by extraction with 0.2 M monoethanolamine as described previously (section D of Materials and Methods). The specific activity of the feed labelled with L-[U-^{14}C]phenylalanine was 492 dpm(mg)$^{-1}$, while that labelled with L-[U-^{14}C]tyrosine was 491 dpm(mg)$^{-1}$.

b. *Administration of Labelled Feed and Collection of Excreta*

Excreta were collected during the administration of labelled feed to rats on the continuous feeding regimen. Collection was not begun until 12 h after the start of providing labelled feed. During the 12 h delay it was expected that unlabelled digesta in the gastrointestinal tract would be largely replaced with digesta derived from labelled feed. Thompson and Hollis (58) have shown that 50 to 60 %

of the radioactivity of ^{106}Ru administered by stomach tube was excreted in the feces of rats within 12 h, and within 15 h more than 80 % of the administered label had been excreted in the feces. ^{106}Ru was used as a marker because of its low absorption from the gastrointestinal tract. Although the complete replacement of gastrointestinal contents may not have occurred within 12 h in the present experiments, a delay of longer than 12 h was not employed in order to minimize the urinary excretion of label from the metabolism of L-[U-^{14}C]phenylalanine and L-[U-^{14}C]tyrosine recycled from body protein.

The normal feed of 4 typical rats on the constant feeding regimen was substituted with the labelled feed so that each labelled amino acid was fed to 2 rats. After the initial 12 h delay, urine and feces were collected for 24 h. Urine was collected in 0.5 N H_2SO_4 in a pan beneath each metabolism cage, and the feces were frequently removed from a screen above the acid. In order to use a minimum volume of acid to collect the urine, dividers were used to reduce the urine collection area of the metabolism cages to approximately 17 × 7 cm. There was no visible contamination of the urine with feed discarded by the rats and no visible loss of urine.

The recovery of label in the feces and urine has been used to correct for unabsorbed L-[U-^{14}C]phenylalanine and L-[U-^{14}C]tyrosine in treatment A and also for urinary excretion of phenylalanine and derivatives of phenylalanine other than those formed from tyrosine in both treatments A and B.

G. Statistical Method

The means of body weights, dry matter intakes, N excretions, growth rates, oxidations of L-[U-^{14}C]phenylalanine and L-[U-^{14}C]-tyrosine as well as protein synthesis, degradation and accretion were compared for statistically significant differences between treatments A and B. Means were compared using Student's *t* as the test criterion and assuming equal variances for any two sets of observations compared.

THEORY

A. Model I.

A simplified model (Fig. 1) of phenylalanine and protein metabolism has been used as a basis for the estimation of the rates of protein synthesis, degradation and accretion. Phenylalanine from the diet has been assumed to enter a single pool from which it may be hydroxylated to form tyrosine, or incorporated into body protein. It has also been assumed that body protein constitutes only a single pool. Although simplified, a model of this nature describing amino N metabolism proved to be of value in the investigations of Picou and Taylor-Roberts (49) and Young et al. (69).

B. Constants, Definitions and Calculations

By estimating the total flux of phenylalanine through the phenylalanine pool as defined by model I, and the fraction of that flux incorporated into body protein, the rate of incorporation of phenylalanine into body protein has been calculated. Corrections have been applied for urinary excretion of phenylalanine and derivatives of phenylalanine other than those formed from tyrosine, and also for endogenous fecal excretion of phenylalanine.

Constants used in the calculations:

Phenylalanine in rat diet: 0.294 mg(mg dietary N)$^{-1}$

Amino acids in rat empty body mass:[1] 6.09 mg(mg body N)$^{-1}$

5.15

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[1] Determined from the amino acid and N analyses of 3 typical rats.

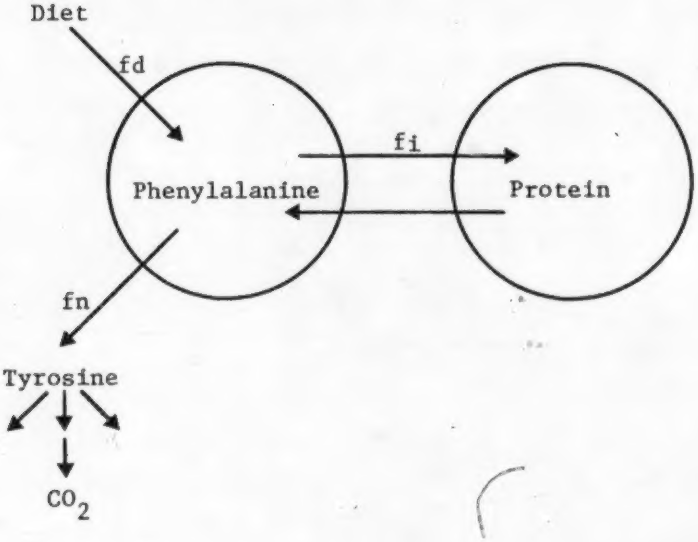
Diet
fd
fi
Phenylalanine
Protein
fn
Tyrosine
CO_2

Fig. 1. Model I, phenylalanine and protein metabolism: fd, rate of absorption of dietary phenylalanine corrected for urinary losses of phenylalanine and derivatives of phenylalanine other than those formed from tyrosine; fn, the in vivo rate of the phenylalanine 4-monooxygenase reaction (hydroxylation of phenylalanine); fi, the rate of phenylalanine incorporation into protein.

Phenylalanine in rat empty body mass:[1] 0.242 mg(mg body N)$^{-1}$

or 0.0397 mg(mg amino acid)$^{-1}$

Symbols are defined as follows:

P - fraction of the ^{14}C of administered L-[U-^{14}C]phenylalanine oxidized to $^{14}CO_2$ at plateau.

T - fraction of the ^{14}C of administered L-[U-^{14}C]tyrosine oxidized to $^{14}CO_2$ at plateau.

ft - total flux leaving the phenylalanine pool by the reactions of hydroxylation and protein synthesis. See equation 8 for the calculation of ft.

M - fraction of ft converted to tyrosine. M was estimated from equation 1:

$$M = \frac{P}{T} \tag{1}$$

DN - N consumed in the diet.

FN - total fecal N excretion.

UN - N excreted in the urine.

EFN - endogenous fecal N excretion. EFN was estimated to be 1.32 g(kg dry matter ingested)$^{-1}$ according to the results of Twombly and Meyer (59) and Mitchell and Bert (42).

EFphe - estimated endogenous fecal phenylalanine losses. EFphe was estimated from EFN and the phenylalanine/N ratio of the empty body mass of typical rats:

$$EFphe = 0.242 \text{ mg(mg body N)}^{-1}(EFN) \tag{2}$$

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[1] Determined from the amino acid and N analyses of 3 typical rats.

G - percent of the ^{14}C of administered L-[U-^{14}C]phenylalanine excreted in the urine as phenylalanine or derivatives of phenylalanine other than those formed from tyrosine. G was estimated from equation 3:

G = percent of dpm of administered L-[U-^{14}C]phenylalanine recovered in the urine (from Table 4) - $\bar{M}$(percent of dpm of administered L-[U-^{14}C]tyrosine recovered in the urine [from Table 4]) (3)

where $\bar{M}$ is the mean value of M from both treatments A and B (from Table 3). The collection of feces and urine for the calculation of G was described in section F of Materials and Methods.

Uphe - urinary excretion of phenylalanine and derivatives of phenylalanine other than those formed from tyrosine. Uphe was estimated from equation 4:

$$\text{Uphe} = 0.294\ \text{mg(mg dietary N)}^{-1}(\text{DN})\left(\frac{G}{100\ \%}\right) \qquad (4)$$

fd - estimated rate of absorption of dietary phenylalanine from the gastrointestinal tract after correcting for urinary losses of phenylalanine and derivatives of phenylalanine other than those formed from tyrosine. It was assumed that the fraction of dietary N excreted in the feces was equivalent to the fraction of dietary phenylalanine not absorbed during digestion as was found by Kuiken and Lyman (37). Dietary N excreted in the feces was estimated as FN minus EFN.

$$fd = 0.294\ \text{mg}(\text{mg dietary N})^{-1}(DN)(1 - \frac{FN - EFN}{DN}) - Uphe \quad (5)$$

r - net retention of phenylalanine by the animal. The value of r was estimated from the net retention of N by each rat as determined by N balance, and from the phenylalanine/N ratio of the empty body mass of typical rats.

$$r = 0.242\ \text{mg}(\text{mg body N})^{-1}(DN - FN - UN + EFN) \quad (6)$$

fn - total in vivo flux of the phenylalanine 4-monooxygenase reaction (hydroxylation of phenylalanine), which was estimated from the following equation:

$$fn = fd - r - EFphe \quad (7)$$

Having defined fn it is now possible to give the equation for the estimation of ft:

$$ft = \frac{fn}{M} \quad (8)$$

fi - rate of phenylalanine incorporation into body protein, which was estimated from equation 9:

$$fi = (1 - M)ft \quad (9)$$

where it was assumed that (1 - M) was equivalent to the fraction of ft that was utilized for protein synthesis. Substituting equations 1, 7 and 8 into equation 9 yields:

$$fi = \frac{(1 - \frac{P}{T})(fd - r - EFphe)T}{P} \quad (10)$$

For the special case of an animal in N balance, r equals zero and equation 10 reduces to:

$$fi = \frac{(1 - \frac{P}{T})(fd - EFphe)T}{P} \tag{11}$$

S - rate of protein synthesis, which was estimated from equation 12:[1]

$$S = \frac{fi}{0.0397\ mg(mg\ amino\ acid)^{-1}} \tag{12}$$

In calculating S from fi the assumption has been made that the phenylalanine content of slowly synthesized proteins is the same as the phenylalanine content of rapidly synthesized proteins. If, for example, rapidly synthesized proteins have a higher phenylalanine content than slowly synthesized proteins, the total rate of protein synthesis would be overestimated.

Z - rate of accretion of protein. Z was estimated from the N balance of each rat and from the N/amino acid ratio of the empty body mass of typical rats:[1]

$$Z = 6.09\ mg(mg\ body\ N)^{-1}(DN - FN - UN + EFN) \tag{13}$$

D - rate of degradation of protein, which was estimated by difference:[1]

$$D = S - Z \tag{14}$$

[1] See footnote, page 30.

C. Correction for Urinary Loss of Phenylalanine and Derivatives of Phenylalanine Other than Those Formed from Tyrosine[1]

G = percent of dpm of administered L-[U-^{14}C]phenylalanine recovered in the urine − $\overline{M}$(percent of dpm of administered L-[U-^{14}C]tyrosine recovered in the urine)

= 5.37 % − 0.462(4.78 %)

= 3.16 %

This value of G has been used in the calculations for all rats. The data used to calculate G is found in Tables 3 and 4.

D. Sample Calculation[1]

The results obtained with a typical rat from treatment A are used for the example. Data from this rat required for the calculations are:

DN	396 mg(day)$^{-1}$
FN	76.0 mg(day)$^{-1}$
EFN	15.5 mg(day)$^{-1}$
UN	205 mg(day)$^{-1}$
EFphe	3.75 mg(day)$^{-1}$
P	0.133
T	0.304

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[1] All calculations were done to 4 figures in order to avoid round-off error. Accordingly, numbers in sections C and D of the Theory have all been calculated to 4 figures before being rounded off to 3 figures for presentation here.

a. *Calculation of Phenylalanine Absorption (fd), Retention (r) and Hydroxylation (fn)*

$$\mathrm{fd} = 0.294\ \mathrm{mg}(\mathrm{mg\ dietary\ N})^{-1}(\mathrm{DN})\left(1 - \frac{\mathrm{FN} - \mathrm{EFN}}{\mathrm{DN}}\right) - \mathrm{Uphe} \qquad (5)$$

$$= 0.294\ \mathrm{mg}(\mathrm{mg\ dietary\ N})^{-1}(\mathrm{DN})\left(1 - \frac{\mathrm{G}}{100\ \%} - \frac{\mathrm{FN} - \mathrm{EFN}}{\mathrm{DN}}\right)$$

$$= 0.294(396)\left(1 - 0.316 - \frac{76.0 - 15.5}{396}\right)\ \mathrm{mg}(\mathrm{day})^{-1}$$

$$= 95.0\ \mathrm{mg}(\mathrm{day})^{-1}$$

$$\mathrm{r} = 0.242\ \mathrm{mg}(\mathrm{mg\ body\ N})^{-1}(\mathrm{DN} - \mathrm{FN} - \mathrm{UN} + \mathrm{EFN})$$

$$= 0.242(396 - 76.0 - 205 + 15.5)\ \mathrm{mg}(\mathrm{day})^{-1}$$

$$= 31.5\ \mathrm{mg}(\mathrm{day})^{-1}$$

$$\mathrm{fn} = \mathrm{fd} - \mathrm{r} - \mathrm{EFphe} \qquad (7)$$

$$= (95.0 - 31.5 - 3.75)\ \mathrm{mg}(\mathrm{day})^{-1}$$

$$= 59.7\ \mathrm{mg}(\mathrm{day})^{-1}$$

b. *Calculation of Fraction (M) of Total Phenylalanine Flux (ft) Converted to Tyrosine and Incorporation of Phenylalanine into Protein (fi)*

In the calculations for treatment A a correction has been applied for the fraction of L-[U-^{14}C]phenylalanine and L-[U-^{14}C]-tyrosine not absorbed from the gastrointestinal tract. The correction has been made by dividing P and T for each rat by the fraction of the [^{14}C]amino acid which was not recovered in the feces (from Table

4).[1] This correction has not been applied for the calculations for treatment B.

$$P' = \frac{0.133}{1 - 0.0367}$$

$$= 0.138$$

$$T' = \frac{0.304}{1 - 0.0496}$$

$$= 0.320$$

where P' and T' are the corrected values of P and T, respectively.

$$M = \frac{P'}{T'} \qquad \text{(from equation 1)}$$

$$= \frac{0.138}{0.320}$$

$$= 0.431$$

$$ft = \frac{fn}{M} \qquad (8)$$

$$= \frac{59.7}{0.431}\ \text{mg(day)}^{-1}$$

$$= 138\ \text{mg(day)}^{-1}$$

$$fi = (1 - M)ft \qquad (9)$$

$$= (1 - 0.431)(138)\ \text{mg(day)}^{-1}$$

$$= 78.7\ \text{mg(day)}^{-1}$$

---

[1] Considering the duration of the experiment described in Table 4, some of the absorbed [^{14}C]amino acid may have been recycled and excreted with the EFN. Although this would tend to cause an overestimation of unabsorbed [^{14}C]amino acid, the error has been presumed to be small, and has not been considered in the calculations.

c. Calculation of Rates of Protein Accretion (Z), Synthesis (S) and Degradation (D)[1]

$$Z = 6.09 \text{ mg(mg body N)}^{-1}(DN - FN - UN + EFN) \qquad (13)$$

$$= 6.09(396 - 76.0 - 205 + 15.5) \text{ mg(day)}^{-1}$$

$$= 794 \text{ mg(day)}^{-1}$$

$$S = \frac{fi}{0.0397 \text{ mg(mg amino acid)}^{-1}} \qquad (12)$$

$$= \frac{78.7}{0.0397} \text{ mg(day)}^{-1}$$

$$= 1980 \text{ mg(day)}^{-1}$$

$$D = S - Z \qquad (14)$$

$$= (1980 - 794) \text{ mg(day)}^{-1}$$

$$= 1190 \text{ mg(day)}^{-1}$$

Z, S and D will also be presented as mg(100 g body wt.)$^{-1}$(day)$^{-1}$.

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[1] Protein synthesis has been calculated as the weight of amino acids incorporated into body protein. This differs from the weight of protein synthesized per unit time by the weight of one water molecule per peptide bond. Similarly, protein accretion has been calculated as the increase in the weight of constituent amino acids, and degradation has been calculated as the weight of amino acids released from protein.

RESULTS

A. Feed Consumption, Growth and N Balance

A significant difference in live body weights at the time of administration of the [^{14}C]amino acids was found between the rats of treatment A (radioisotope administered with the feed) and treatment B (radioisotope administered by constant infusion) (Table 1). There was also a small but significant difference in dry matter consumption between the two groups. Both dry matter consumption and live body weights were greater for the rats of treatment A than the rats of treatment B. There was, however, no significant difference in weight gain over the entire experimental period.

Because dry matter consumption was significantly different, N consumption was also significantly different (Table 2). In addition, N excreted in the feces and N excreted in the urine were significantly different between treatments A and B, with the rats of treatment A consuming and excreting more N than the rats of treatment B. Although statistically significant, it was felt that the differences in N balance were probably not large enough to confound any significant effect of the experimental treatments.

B. Oxidation of L-[U-^{14}C]Phenylalanine and L-[U-^{14}C]Tyrosine

During the administration of L-[U-^{14}C]phenylalanine and L-[U-^{14}C]-tyrosine, the expiration of $^{14}CO_2$ reached plateau levels sooner with treatment B than with treatment A (Fig. 2). It also appears that L-[U-^{14}C]tyrosine tended to establish a plateau sooner than L-[U-^{14}C]-

Table 1. Feed intake and growth data. N balance trials were performed on individual animals for 12 to 15 days. During this time daily feed consumption was measured and the rats were weighed every 3 or 4 days. L-[U-^{14}C]tyrosine or L-[U-^{14}C]-phenylalanine was administered at day 4, 5 or 6 and the remaining [^{14}C]amino acid was administered at day 8, 9 or 10. There were always 3 or more days between administration of [^{14}C]amino acids. The radioisotopes were administered either with the diet (treatment A) or by constant infusion (treatment B), and rate of expiration of $^{14}CO_2$ was measured. Body weights were interpolated to be intermediate between the times of administration of L-[U-^{14}C]phenylalanine and L-[U-^{14}C]tyrosine. Mean values with standard errors are given and numbers of observations are provided in parentheses.

Treatment	No. of animals	Live body weight g	Dry matter consumption g(day)$^{-1}$	Weight gain (calculated from initial and final body weights) g(day)$^{-1}$
A	6	160±5.6(6)*	12.4±0.066(64)*	3.0±0.15(6)
B	5	123±2.7(5)*	12.0±0.058(52)*	2.9±0.23(5)

*A and B significantly different ($P < 0.05$)

Table 2. N balance data. Experimental details are described in Table 1. Daily collections of feces and urine were performed on individual animals. Total N was determined on each collection of feces and urine. Mean values with standard errors are given and numbers of observations are provided in parentheses.

Treat-ment	No. of animals	N consumption $mg(day)^{-1}$	N excreted in feces $mg(day)^{-1}$	N excreted in urine $mg(day)^{-1}$
A	6	418±2.2(64)*	82.2±1.1(64)*	209±3.0(64)*
B	5	402±2.0(52)*	74.8±1.2(52)*	190±3.8(52)*

*A and B significantly different ($P < 0.05$)

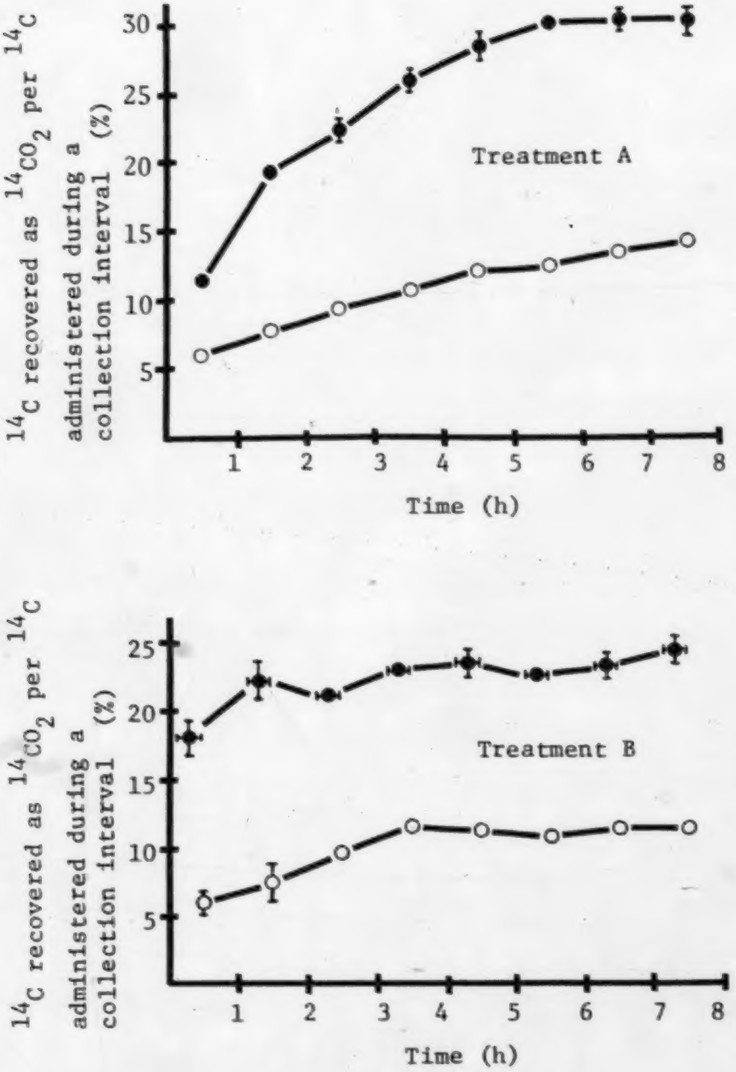

Fig. 2. Expiration of $^{14}CO_2$ after the start of administration of [^{14}C]amino acids. $^{14}CO_2$ was collected for 30 min of each hour. Points are positioned midway through the collections periods. Standard errors larger than the diameter of the spots are indicated by vertical bars. Horizontal bars indicate variations in the time of observation. Treatment A; all points are the means of observations of 5 or 6 rats. Treatment B; points up to 3 h are the means of observations of 3, 4 or 5 rats, while all points after 3 h are the means of 5 rats. ●, L-[U-^{14}C]tyrosine; ○, L-[U-^{14}C]phenylalanine.

phenylalanine, for which a true plateau may not have been reached in treatment A.

All plateau values in treatment B were estimated from the 3 observations between 5 and 8 h after the start of infusion. In treatment A, the rat chamber was designed so that there would be a minimum wastage of labelled feed. Consequently, the rat had a limited space in which to move around, and when the rat was active, the expiration of $^{14}CO_2$ tended to be greater than when it was resting. Therefore, the observations taken while the rat was visibly active were discarded. In order to assure that each plateau value was estimated from a minimum of 3 observations, plateau values in treatment A were estimated as the mean of observations of expired $^{14}CO_2$ between 4 and 8 h after the start of feeding the labelled feed.

In both treatments, A and B, the rate of oxidation of L-[U-^{14}C]-phenylalanine was less than half of the rate of oxidation of L-[U-^{14}C]-tyrosine (Table 3). The rate of oxidation of both amino acids was significantly less in treatment B than in treatment A.

Generally, the extent of oxidation of [^{14}C]phenylalanine in rats has been found (1, 20, 56, 57) to be greater than that observed in the present experiments. However, expired $^{14}CO_2$ has usually been collected for 24 h following a single injection of labelled phenylalanine. As much as 38 % of the administered ^{14}C was recovered in $^{14}CO_2$ expired in 24 h after an intraperitoneal injection of DL-[1-^{14}C]-phenylalanine (56, 57). A longer time allowing for recycling of [^{14}C]phenylalanine from body protein compared to the present experiments may have contributed to the extent of oxidation recorded.

Table 3. Oxidation of L-[U-^{14}C]phenylalanine and L-[U-^{14}C]tyrosine. Experimental details are given in Table 1 and in the text. Plateau values for the administration of L-[U-^{14}C]tyrosine and L-[U-^{14}C]phenylalanine were determined once for each rat. The fraction of phenylalanine converted to tyrosine (M) has been corrected for unabsorbed [^{14}C]amino acid in treatment A as explained in the Sample Calculation. Mean values with standard errors are given and numbers of observations are provided in parentheses.

		Expiration of $^{14}CO_2$ at plateau		
Treatment	No. of animals	[^{14}C]phenylalanine (% of ^{14}C administered) (P × 100%)	[^{14}C]tyrosine (% of ^{14}C administered) (T × 100%)	M
A	6	12.8±0.30(6)*	28.4±0.58(6)*	0.444±0.010(6)
B	5	11.2±0.18(5)*	23.4±0.74(5)*	0.480±0.026(5)

*A and B significantly different ($P < 0.05$)

Godin and Dolan (21) reported values of 14 % of the ^{14}C of DL-[1-^{14}C]-phenylalanine and 10 % of the ^{14}C of DL-[3-^{14}C]phenylalanine recovered in the $^{14}CO_2$ expired in 24 h following intravenous injection. These are the lowest values noted in the literature and are similar to those reported here.

Phenylalanine intake and the route of administration of [^{14}C]-phenylalanine, but not the stereoisomerism of the labelled phenylalanine, appear to influence the rate of oxidation to $^{14}CO_2$. DL-[1-^{14}C]phenylalanine was oxidized to the same extent as L-[1-^{14}C]-phenylalanine (20), indicating that D- and L-phenylalanine are metabolized similarly. Godin and Dolan (21) have shown that reduced dietary phenylalanine intake resulted in a reduced 24 h oxidation of DL-[3-^{14}C]phenylalanine in rats receiving intravenous injections of labelled phenylalanine. In general, the results in the literature also indicate that intraperitoneal injection of labelled phenylalanine results in greater oxidation than does intravenous injection. Considering the range of results reported in the literature, and the possible effects of method and route of administration on the oxidation of labelled phenylalanine, as well as the effects of phenylalanine intake, the relatively low values observed in the present experiments appear acceptable.

Godin and Dolan (21) investigated the oxidation of intravenously injected DL-[2-^{14}C]tyrosine in rats. Approximately 31 % of the injected ^{14}C was recovered in the 24 h collection of expired $^{14}CO_2$. Since the factors affecting the oxidation of labelled phenylalanine may also affect the oxidation of labelled tyrosine, the agreement with the results presented in this thesis may only be fortuitous. Nevertheless, the relative extents of oxidation of labelled tyrosine and labelled phenylalanine found by Godin and Dolan (21) agree well with the relative rates of oxidation reported here.

C. Urinary and Fecal Excretion of ^{14}C Following Administration of L-[U-^{14}C]Tyrosine and L-[U-^{14}C]Phenylalanine with the Diet

In order to correct for unabsorbed [^{14}C] amino acid in treatment A and also for urinary losses of phenylalanine and phenylalanine derivatives not formed from tyrosine in treatments A and B, ^{14}C excreted in the urine and feces was recovered during the continuous feeding of the diet labelled with L-[U-^{14}C]tyrosine or L-[U-^{14}C]-phenylalanine (Table 4). Approximately 5 % of the radioactivity of L-[U-^{14}C]tyrosine and 4 % of the radioactivity of L-[U-^{14}C]phenylalanine was recovered in the feces, and about 5 % of each was found in the urine. The calculation of the rate of protein synthesis was corrected for these losses as described in the Theory.

Following the inclusion of L-[U-^{14}C]phenylalanine in a meal fed to rats, Aguilar et al. (1) recovered 1.3 % of the absorbed radioactivity in a 24 h urine collection. In the same study, less than 3 % of the label consumed with the diet was recovered in the feces, gastrointestinal contents and spilled feed. Approximately 4 % of intravenously injected radioactivity of DL-[3-^{14}C]phenylalanine or DL-[1-^{14}C]phenylalanine was recovered in 24 h urine collections in the experiments of Godin and Dolan (21). Godin and Dolan (21) also found 8 % of the injected radioactivity of DL-[2-^{14}C]tyrosine in the urine.

Table 4. Urinary and fecal excretion of radioactivity following administration of L-[U-^{14}C]tyrosine and L-[U-^{14}C]-phenylalanine with the diet. Feed labelled with L-[U-^{14}C]-tyrosine and L-[U-^{14}C]phenylalanine was continuously fed to rats in the same manner as unlabelled feed. After 12 h, urine and feces were collected for the next 24 h. The estimated error in absorption of phenylalanine (fd) attributed to the urinary excretion of phenylalanine and derivatives of phenylalanine other than those formed from tyrosine was calculated from the urinary excretion of ^{14}C as described in the Theory. Mean values with ranges are given and numbers of observations are provided in parentheses.

Labelled amino acid in feed	No. of animals	Dpm recovered in urine (% of dpm administered)	Dpm recovered in feces (% of dpm administered)
L-[U-^{14}C]tyrosine	2	4.78±1.06(2)	4.96±0.76(2)
L-[U-^{14}C]phenylalanine	2	5.37±1.25(2)	3.67±0.81(2)

The results of Godin and Dolan (21) and Aguilar et al. (1) are consistent with those presented in this thesis.

D. Parameters of Phenylalanine Metabolism

The results of the N balance analyses and the oxidations of L-[U-^{14}C]phenylalanine and L-[U-^{14}C]tyrosine have been used to estimate the net retention (r), absorption (fd), hydroxylation (fn) and total flux (ft) of phenylalanine, in addition to its incorporation into body protein (fi) (Table 5). An example of the calculations has been provided in the Sample Calculation.

E. Rates of Protein Synthesis (S), Accretion (Z) and Degradation((D)

The rates of protein synthesis and degradation (Table 6) in the whole body were calculated from the results of the present experiments. Significant differences were found between treatments A and B in the rates of synthesis and accretion (Table 6). Since accretion is estimated from nitrogen balance, there would not be an effect of route of administration of label on these values. Of the factors used to calculate protein synthesis, however, P, T and M could have been affected by treatment. Although P and T were significantly different between treatments, the ratios of P/T, i.e. M, were not (Table 3). Since M was not significantly different between treatments, the differences in the rates of protein synthesis calculated from M would be due to factors other than route of administration of the labelled amino acids.

Factors which could have contributed to the differences in

Table 5. Parameters of phenylalanine metabolism. Experimental details are provided in Table 1. The parameters of phenylalanine metabolism have been calculated according to the method in the Theory. Mean values with standard errors are given and numbers of observations are provided in parentheses.

Treatment	No. of animals	Accretion (r) mg(day)$^{-1}$	Absorption (fd) mg(day)$^{-1}$	Hydroxylation (fn) mg(day)$^{-1}$	Total flux (ft) mg(day)$^{-1}$	Incorporation into protein (fi) mg(day)$^{-1}$
A	6	34±1.9(6)	100±1.1(6)	61±1.6(6)*	138±4.2(6)*	77±3.3(6)*
B	5	37±1.3(5)	97±1.3(5)	56±0.8(5)*	118±5.4(5)*	62±5.1(5)*

*A and B significantly different ($P < 0.05$)

accretion and synthesis between treatments may have been body weight and dry matter, or N, consumption. The rates of protein synthesis, accretion and degradation have been expressed both as mg(day)$^{-1}$ and as mg(100 g body wt.)$^{-1}$(day)$^{-1}$ (Table 6). When expressed as mg(100 g body wt.)$^{-1}$(day)$^{-1}$ the rates of accretion were significantly different between treatments A and B, but the rates of synthesis were not. When expressed as mg(day)$^{-1}$ the rates of synthesis were significantly different between treatments, and the rates of accretion were not. Since the rates of protein synthesis in treatments A and B were very similar per unit of body weight, the whole animal rate of synthesis appears to have been predominantly a function of the mass of the animal. On the other hand, the total rates of protein accretion for the rats were not different for treatments A and B despite differences in body weight. In treatments A and B, respectively, 2.1 and 2.3 mg of body protein accrued per mg of N consumed, indicating that the accretion of protein by the rats may have been predominantly a function of dietary N intake rather than a function of body mass.

Degradation has been estimated as the difference between synthesis and accretion. When expressed per unit of body weight degradation tended to be larger for treatment A than for treatment B, showing that the lower rate of protein accretion per unit of body weight in treatment A correlated with a greater rate of protein degradation. Since degradation was determined by difference, the standard errors for the estimation of degradation are larger than the standard errors for the corresponding estimates of synthesis and

Table 6. Rates of protein synthesis (S), accretion (Z) and degradation (D). Experimental details are provided in Table 1. The rates of protein synthesis, accretion and degradation have been calculated according to the method presented in the Theory.[1] Mean values with standard errors are given and numbers of observations are provided in parentheses.

		Synthesis		Accretion		Degradation	
Treat- ment	No. of animals	$mg(day)^{-1}$	$mg(100\ g\ body\ wt.)^{-1}(day)^{-1}$	$mg(day)^{-1}$	$mg(100\ g\ body\ wt.)^{-1}(day)^{-1}$	$mg(day)^{-1}$	$mg(100\ g\ body\ wt.)^{-1}(day)^{-1}$
A	6	1941±85(6)*	1216±55(6)	866±47(6)	549±50(6)*	1075±117(6)*	667±63(6)
B	5	1561±130(5)*	1255±81(5)	936±33(5)	757±25(5)*	626±132(5)*	499±98(5)

[1] See footnote, page 30.

*A and B significantly different ($P < 0.05$)

accretion. This appears to have obscured the statistical significance when degradation was expressed as mg (100 g body wt.)$^{-1}$(day)$^{-1}$. On a whole body basis degradation varied with synthesis, resulting in little difference in accretion (expressed as mg[day]$^{-1}$) between treatments A and B.

DISCUSSION

A. Diurnal Variation

Diurnal variation may have had a significant effect on the estimation of the rates of protein synthesis, accretion and degradation. Although the values for phenylalanine absorption (fd) and retention (r) were estimated from the entire daily consumption and excretion of N and phenylalanine, the value of M was estimated from measurements taken only at one time of the day. If the value of M does not reflect the fraction of the total daily flux of phenylalanine hydroxylated to tyrosine, then the calculated rates of protein synthesis, accretion and degradation will also not reflect the total daily values. From equations 8 and 9 (section B of Theory) it is apparent that if M was underestimated then the rate of phenylalanine incorporation into protein (fi) was overestimated, and if M was overestimated then fi was underestimated.

In the experiments described in this thesis, continuous feeding was used in an attempt to keep the rate of oxidation of L-[U-^{14}C]-phenylalanine and L-[U-^{14}C]tyrosine constant during the periods of isotope infusion, and also in an attempt to minimize diurnal variations in fd, r and fi. Although plateaus in the expiration of $^{14}CO_2$ were established, the recent work of Obled, Arnal and Fauconneau (47) indicates that diurnal variation in fi was not significantly reduced by continuous feeding. In their experiments, measurements of protein synthesis were taken once during the night and once during the day on liver proteins and proteins of the pooled intestines, pancreas and mesentry. Rats were either fed ad libitum or received a meal every 4 h. Although there was a reduction in the diurnal variation as a result of meal feeding, it was minor. The day/night ratio for the rate of liver protein synthesis was 0.49 for the ad libitum fed rats and 0.58 for the meal fed rats. Similar results were obtained for the other viscera, As a result of diurnal variation, then, the rate of fi determined in the present experiments at the time of isotope administration may have been different from the average daily rate.

In addition to diurnal variation of fi, the estimated value of fi may not reflect the actual value at the time of isotope administration. This is apparent from an examination of equation 10 (section B of Theory). Assuming for the moment that endogenous fecal phenylalanine excretion (EFphe) was negligible compared to fd, and substituting equation 1 into equation 10, we have:

$$fi = \frac{(1 - M)(fd - r)}{M} \qquad (15)$$

Since the feeding regimen of the rats was continued throughout the infusion of labelled amino acids, the estimate of fd determined from daily measurements probably reflects the value of fd at the time of isotope infusion. From equation 15 it is apparent that if the value of r at the time of isotope infusion deviated from the measured daily value of r, then the calculated value of fi would be an inaccurate estimate of fi at the time of infusion. Since the value of r was approximately one third the value of fd (Table 5), diurnal variation in r could indeed have had a significant effect on the estimated value of fi. Thus, the value of fi calculated from the experiments presented in this thesis was an estimate of the rate of fi at the time of isotope infusion, but was subject to an error resulting from diurnal variations in r.

B. *Continuous Feeding Versus Ad Libitum Feeding*

In the investigations of Obled, Arnal and Fauconneau (47) meal fed rats consumed 10 % less feed but grew at the same rate as ad libitum fed rats. In addition, the mean daily rate of protein synthesis for all organs studied was 32 % less for the meal fed rats than for the ad libitum fed rats. Although a difference in feed consumption confuses the interpretation of these results, they indicate that continuous feeding, such as in the present experiments, may have resulted in a lower rate of protein synthesis than would have occurred with ad libitum feeding.

As a consequence of the procedure of continuous feeding, the rats in the present experiments received only 66 % of the feed consumed by ad libitum fed rats. In studies with humans, Young et al. (69) found that the rate of protein synthesis in the whole body was a constant function of the dietary protein consumption in infants, young adults and elderly persons. If this relationship holds for restricted feed intake in rats, the rates of protein synthesis estimated in the present experiments may have been low compared to rats consuming more feed.

C. Model II

An estimate of the error resulting from the use of a simplified model of phenylalanine and protein metabolism (Fig. 1) may be obtained by applying the calculations described in the Theory to a more realistic model (Fig. 3). In the rat, most phenylalanine 4-monooxygenase activity was found in the liver and a smaller amount was found in the kidney (43). No activity was found in the pancreas, brain, spleen, heart or muscle (43). These results indicate that there are only two free phenylalanine pools (kidney and liver) which serve as precursors for tyrosine. From the sum of these pools (B) a weighted mean specific activity (b) for that phenylalanine which was the precursor of tyrosine may be calculated.

$$B = B' + B''$$

where B' and B" are free phenylalanine pools.

$$k_1 = \frac{k_1'B' + k_1''B''}{B}$$

where k_1, k_1' and k_1'' are assumed to be first order rate constants for B, B' and B", respectively.

$$b = \frac{k_1'B'b' + k_1''B''b''}{k_1B}$$

where b' and b" are the specific activities of the free phenylalanine pools.

There are an unknown number of free phenylalanine pools which

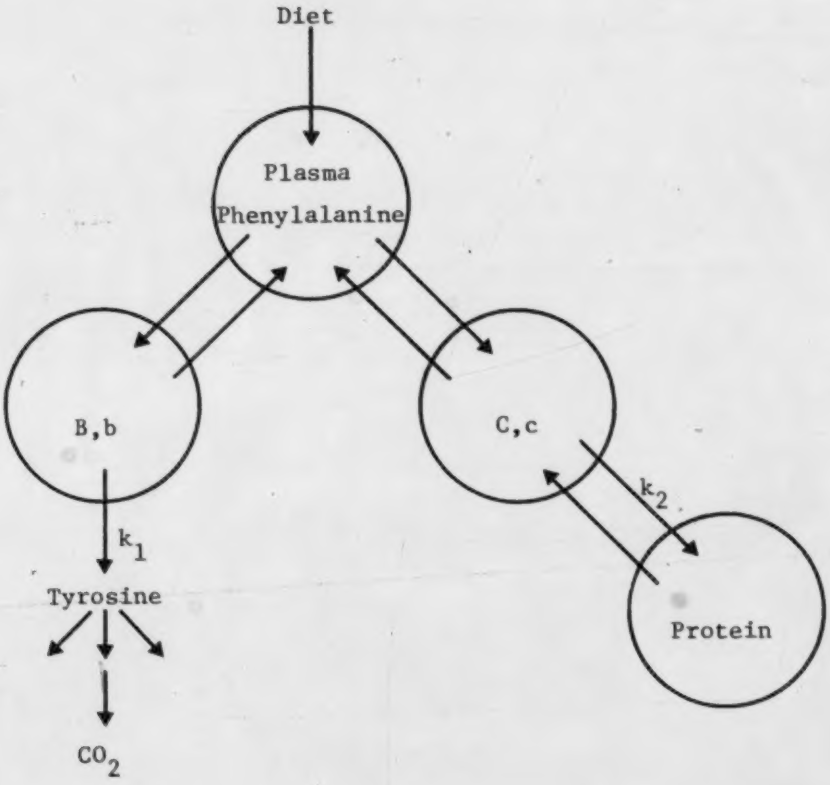
Diet
Plasma
Phenylalanine
B,b
C,c
k_1
k_2
Tyrosine
CO_2
Protein

Fig. 3. Model II, phenylalanine and protein metabolism: B and C, phenylalanine pools; b and c, specific activities of the phenylalanine pools; k_1 and k_2 are assumed to be first order rate constants. Note: since liver and kidney free phenylalanine pools may be common precursors for both protein synthesis and tyrosine synthesis, B and C may not be mutually exclusive as suggested by the diagram.

serve as precursors of protein synthesis. However, if C represents the sum of these pools and c represents the weighted mean specific activity, C, c and k_2 may be defined in a manner analogous to B, b and k_1. Model II, therefore, may be used to predict errors resulting from the use of a single-pool model (model I) in describing phenylalanine metabolism.

From model II:

$$fn = k_1B$$

$$ft = k_1B + k_2C$$

where k_2 is the rate constant for the incorporation of phenylalanine into body protein, assumed to be first order.

$$M = \frac{fn}{ft} \qquad \text{(from equation 8)}$$

$$= \frac{k_1B}{k_1B + k_2C} \qquad (16)$$

As would be determined experimentally from model II (denoted by an asterisk):

$$M^* = \frac{k_1Bb}{k_1Bb + k_2Cc} \qquad (17)$$

Therefore, $M^* = M$ only if $b = c$.

If we let $x = \frac{k_2C}{k_1B}$ and $y = \frac{c}{b}$

then
$$M^* = \frac{k_1 Bb}{k_1 Bb + xyk_1 Bb}$$
$$= \frac{1}{1 + xy}$$

$$fi^* = \frac{(1 - \frac{1}{x + xy})fn}{\frac{1}{1 + xy}}$$
$$= xyfn$$
$$= \frac{c}{b}k_2 C$$
$$= \frac{c}{b}fi \qquad (18)$$

It appears, then that the estimated rate of incorporation of phenylalanine into body protein was a linear function of the ratio, c/b. If c/b = 1 then fi* = fi and the "true" rate of phenylalanine incorporation as derived from model II could have been calculated from model I. However, in the more likely case the c/b ≠ 1, the rate of phenylalanine incorporation calculated from model I would have been in error by a factor of b/c because the assumption that c = b was inherent in the use of model I. If the difference between c and b was small, then the error introduced in the estimation of fi would also be small. Unfortunately, though, neither c or b can be precisely determined.

Since L-[U-^{14}C]phenylalanine was infused into plasma, neither c or b could have been greater than plasma free phenylalanine specific activity. However, because of the intracellular dilution of the label from protein catabolism, the specific activities at the sites of protein synthesis and hydroxylation may have been considerably less than plasma specific activity. The specific activities of free amino acids within tissues has been measured in other studies following continuous infusion of labelled amino acids (17, 18, 67). At plateau, the specific activities of free lysine and tyrosine in liver were found to be 40 and 50 % of plasma free lysine and tyrosine, whereas the specific activities of free lysine in muscle was 65 to 70 % of plasma (18). Similar results have been reported for glycine (17). Other workers (67) have found the specific activities of liver and muscle free lysine to be 63 and 88 % of plasma, respectively.

Approximate values for c and b may be postulated in order to estimate the error which may have resulted from the use of model I. Airhart et al. (2) have isolated valine from liver valyl-tRNA after intraperitoneal injection of [^{3}H]valine. Since amino acyl-tRNA is the amino acid precursor of protein synthesis, the specific activity of valine isolated from valyl-tRNA should reflect the specific activity of the liver free valine pool supplying valine for protein synthesis. The specific activity was found to be intermediate between plasma and liver free valine. If the same relationship holds for phenylalanine and for tissues other than liver, the value of c may have been between 70 and 90 % of plasma free phenylalanine specific activity (remembering that c is a weighted mean for all tissues and that muscle protein synthesis comprises a significant proportion of total body protein synthesis [4, 67]). If b was also intermediate between plasma and tissue specific activities, a value for b of 60 to 90 % of plasma free phenylalanine specific activity may have been realistic. If this was the case, then the experimentally determined value of fi using calculations derived from model I should be in the range of 0.8 to 1.5 times the value of fi obtained using model II. If b was less than 60 % of plasma, a value for fi greater than 150 % of the value derived from model II could have resulted. If b was more than 90 % of plasma, a maximum underestimation of fi (b equal to 100 % of plasma) equal to 70 % of the value derived from model II could have occurred.

D. Error Resulting from the Intravenous Infusion of L-[U-^{14}C]Tyrosine

In order to estimate a value for M, both P and T were determined by the continuous infusion of L-[U-^{14}C]phenylalanine and L-[U-^{14}C]-tyrosine, respectively. In doing so, an assumption has been made that L-[U-^{14}C]tyrosine produced from the endogenous hydroxylation of L-[U-^{14}C]phenylalanine was oxidized to the same extent as intravenously infused L-[U-^{14}C]tyrosine. Because tyrosine is utilized for a variety of anabolic functions including synthesis of melanin, catecholamines, thyroid hormones and protein as well as catabolism to fumarate and acetoacetate (39), this assumption may not be correct. Because of tissue variations in the relative rates of the various metabolic routes of tyrosine, the proportion of endogenously synthesized L-[U-^{14}C]tyrosine which was oxidized to $^{14}CO_2$ in the liver and kidney (the only tissues possessing phenylalanine 4-monooxygenase activity [43]) may not have equaled the proportion of plasma L-[U-^{14}C]tyrosine oxidized to $^{14}CO_2$.

The magnitude of an error which may have occurred due to the difference in the metabolism of plasma L-[U-^{14}C]tyrosine and endogenously synthesized L-[U-^{14}C]tyrosine is obscured by the variety of routes by which tyrosine may be metabolized. The first reaction in the oxidative metabolism of tyrosine is transamination, catalyzed by tyrosine aminotransferase (L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.5) (39). The specific activity of this enzyme is greatest in liver tissue, although significant levels have also been found in heart muscle, muscle (not specified), kidney and brain (15, 61). Liver tissue also catalyses the conversion of tyrosine to homogentisic acid and subsequently acetoacetate (14, 36), but there is conflicting evidence as to the effectiveness of skeletal muscle, heart muscle, smooth muscle and kidney in catalyzing the same conversion (6, 14). Nevertheless, *p*-hydroxyphenylpyruvic acid produced from the transamination of tyrosine in extrahepatic tissues may be transported to the liver for oxidation. Since plasma tyrosine is available to tissues other than liver and these tissues may metabolize tyrosine both anabolically (mainly protein synthesis) and catabolically (transamination), a quantitative prediction of the error in the present experiments resulting from the extrahepatic or extrarenal metabolism of L-[U-^{14}C]tyrosine is not possible. However, if plasma L-[U-^{14}C]tyrosine was oxidized to a lesser extent than L-[U-^{14}C]tyrosine synthesized from L-[U-^{14}C]phenylalanine in the liver and kidney, then an overestimation of the fraction of the total phenylalanine flux converted to tyrosine (M) would have occurred. As a result, the rate of incorporation of phenylalanine into body protein (fi) would have been underestimated. On the other hand, if plasma L-[U-^{14}C]tyrosine was oxidized to a greater extent than

L-[U-^{14}C]tyrosine synthesized in the liver and kidney, then an underestimation of M and an overestimation of fi would have occurred.

An indication of the presence and direction of an error resulting from the difference in metabolism of plasma L-[U-^{14}C]tyrosine and endogenously synthesized L-[U-^{14}C]tyrosine has been obtained by varying the route of administration of L-[U-^{14}C]tyrosine in the present experiments. L-[U-^{14}C]Tyrosine administered with the diet would likely be more available to liver metabolism than L-[U-^{14}C]-tyrosine administered intravenously. A smaller proportion of intravenously administered L-[U-^{14}C]tyrosine would probably reach the liver as a result of being initially available to extrahepatic tissues. Since the majority of endogenous tyrosine synthesis probably occurs in the liver, dietary L-[U-^{14}C]tyrosine may be more representative of endogenously synthesized L-[U-^{14}C]tyrosine than plasma L-[U-^{14}C]-tyrosine. Thus, since L-[U-^{14}C]tyrosine administered with the diet was oxidized to $^{14}CO_2$ to a greater extent than L-[U-^{14}C]tyrosine administered intravenously (Table 3), an underestimation of fi probably resulted.

E. *Comparison of the Present Results with Those Reported in the Literature*

The mean rate of incorporation of amino acids into protein per unit of body weight in the present experiments was 12 g(kg)$^{-1}$(day)$^{-1}$. Waterlow and Stephen (66, 67) reported rates of protein synthesis of 25 to 83 g(kg)$^{-1}$(day)$^{-1}$, and Arnal, Fauconneau and Pech (4) estimated a rate of 32 $g(kg)^{-1}(day)^{-1}$.[1]

The difference between the present results and those reported in the literature may be partially explained by the use of different feeding regimens. In the experiments of Waterlow and Stephen (66, 67) and Arnal, Fauconneau and Pech (4) rats were fed ad libitum up to the time of injection or infusion of radioisotope. In the experiments of Waterlow and Stephen (66, 67) the rats did not have access to feed after the beginning of constant infusion of L-[U-^{14}C]lysine, but in the experiments of Arnal, Fauconneau and Pech (4) feed was offered ad libitum both before and after the injection of L-[U-^{14}C]-lysine. Since both continuous feeding and restricted feeding such as used in my experiments appears to reduce rate of protein synthesis compared to ad libitum feeding (see section B of Discussion), the relatively low rate of protein synthesis found in the present experiments may have been at least partially due to the feeding regimen.

Other factors contributing to the difference between my results and those found in the literature may be inherent inadequacies in the methods used. As explained in the Literature Review, an underestimation of the specific activity of the precursor of protein synthesis may have caused an overestimation of the rate of protein synthesis in the experiments of Waterlow and Stephen (67) and Arnal, Fauconneau and Pech (4). As discussed in sections A, C and D of the

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[1] The time of day during which measurements were taken for the estimation of the rates of protein synthesis were not specified by Waterlow and Stephen (66, 67) and Arnal, Fauconneau and Pech (4), but presumably was during daylight hours.

Discussion, errors due to diurnal variation in M, fi and r, a lack of knowledge of the precursor specific activity and differences between the metabolism of administered L-[U-^{14}C]tyrosine and endogenously produced L-[U-^{14}C]tyrosine may have occurred in the present experiments. The net result of errors of these types may have contributed to the difference in estimated rates of protein synthesis.

F. Protein Degradation

In the studies presented here, protein degradation has been estimated as the difference between synthesis and accretion. The estimate of protein degradation, therefore, is subject to an accumulation of errors. It seems likely, though, that inaccuracies in the N balance measurements, and consequently the estimate of accretion, were relatively minor compared to inaccuracies in the estimate of daily protein synthesis. Thus, inaccuracies in the estimation of protein degradation were likely essentially similar to those of the estimation of synthesis. If, as appears likely, total daily protein synthesis has been underestimated, then daily protein degradation has probably also been underestimated.

A significant proportion of body protein turnover may occur as a result of the extracellular degradation of protein. Although a portion of N excreted in feces is derived from endogenous sources, the majority of protein excreted or sloughed into the gastrointestinal tract is digested and reabsorbed (44, 59). Since amino acids produced from the digestion of endogenous protein mix with dietary and plasma amino acids, the method of estimating protein degradation presented here would include the degradation of endogenous protein in the gastrointestinal tract. Twombly and Meyer (59) estimated that rats fed a 15 % protein diet twice a day secreted or sloughed about 250 mg of protein per day into the gastrointestinal tract. The weights of the rats used in the studies of Twombly and Meyer (59) were comparable to those used in treatment A reported in this thesis, and they consumed approximately the same amount of feed. It may be estimated, then, that the rate of degradation of endogenous protein in the gastrointestinal tract was approximately one quarter of the total estimated rate of degradation for the rats of treatment A.

In the present experiments, the rate of protein degradation for rats of 123 g body weight was 40 % of the rate of protein synthesis, and was 55 % for rats of 160 g body weight. In the experiments of Arnal, Fauconneau and Pech (4) rats which weighed about 200 g were growing at a rate of 4.2 g$(\text{day})^{-1}$. According to the relationship between growth and protein accretion reported in the present experiments, a growth rate of 4.2 g$(\text{day})^{-1}$ would be equivalent to a rate of protein accretion of 6.3 g$(\text{kg})^{-1}(\text{day})^{-1}$. Since Arnal, Fauconneau and Pech (4) reported a rate of protein synthesis of 32 g$(\text{kg})^{-1}(\text{day})^{-1}$, the rate of protein degradation in their experiments might have been about 80 % of the rate of synthesis. A major difference between the treatment of rats in the present experiments and in the experiments of Arnal, Fauconneau and Pech (4) was the feeding regimen. Hence, the efficiency of protein accretion, in terms of protein accrued per unit of protein synthesized, may be greater for continuously fed rats than for ad libitum fed rats. However, one should not ignore the possibility that as rats become larger and, presumably, more mature degradation accounts for a greater proportion of synthesis.

It is apparent that the rate of protein degradation is also a significant component of the energetic efficiency of protein production in growing animals. In studies of muscle protein turnover with growing chicks, Buttery, Boorman and Barrat (12) have reported that the energetic cost of peptide bond synthesis for daily protein deposition was only 56 to 58 % of the cost of peptide bond synthesis attributed to protein turnover. Milligan (40) has pointed out that the measured energetic efficiency of protein production in growing animals has usually been considerably lower than would be predicted from the cost of peptide bond synthesis. It was suggested (40) that the energy requirements of protein turnover may contribute to the difference.

G. Modifications to the Method Presented in this Thesis

a. Substitution of L-[1-^{14}C]Lysine for L-[U-^{14}C]Phenylalanine

It is possible that error that may be inherent in the present method entailing intravenous infusion of L-[U-^{14}C]tyrosine to estimate endogenous tyrosine metabolism might be overcome by substituting L-[1-^{14}C]lysine for L-[U-^{14}C]phenylalanine. The theory, logic and calculations employed for the estimation of the rates of protein synthesis, accretion and degradation would be basically the same as those already described for L-[U-^{14}C]phenylalanine, except that the expiration of $^{14}CO_2$ after the administration of L-[1-^{14}C]lysine may be taken as a direct measure of the fraction of the total lysine flux catabolized, thus eliminating the need for the administration of a second labelled compound.

As with L-[U-^{14}C]phenylalanine, the use of L-[1-^{14}C]lysine requires the existence of only one major route of catabolism of the amino acid. Except for the formation of minor amounts of pipecolic acid in the rat, a search of the literature has revealed only two possible routes of the metabolism of carbon atom 1 of lysine in animals, these being oxidation to CO_2, or incorporation into protein. Intermediates in the major pathway of lysine catabolism in animals are saccharopine, α-aminoadipic acid, α-ketoadipic acid and glutaryl-coenzyme A (7, 26, 27, 31, 32, 38, 51). Carbon atom 1 of lysine is oxidized to CO_2 as a result of the oxidative decarboxylation of α-ketoadipic acid (7, 46).

A minor catabolic route of lysine leads to the formation of pipecolic acid. Pipecolic acid appears to be metabolically inert in the rat, and is excreted in the urine (8, 25). The formation of pipecolic acid has been shown to be relatively unimportant with respect to the quantitative metabolism of L-lysine. After a single injection of L-[U-^{14}C]lysine into gnotobiotic rats, only 3.2 % of injected radioactivity was found as pipecolic acid in urine collected for 6 h (8). The catabolism of L-lysine in chickens appears to be similar to that in rats except that pipecolic acid is converted to α-aminoadipic acid thus entering the major catabolic route of L-lysine (28).

D-Lysine has been shown to be metabolized in a manner quantitatively different from L-lysine. Grove and co-workers (25, 26) using

D- and L-lysine labelled with ^{15}N or ^{14}C found that D-lysine was more readily converted to pipecolic acid than L-lysine in the intact rat. In addition, D-lysine was more readily excreted unchanged in the urine than L-lysine, and D-lysine was not significantly oxidized to CO_2, while L-lysine was readily oxidized. Because of the differences in metabolism, L-[1-^{14}C]lysine and not DL- or D-[1-^{14}C]lysine should be used for the estimation of protein synthesis.

When 4 µC of DL-[6-^{14}C]lysine and 332 mg of unlabelled formate were administered to a rat, formate with a specific activity of 339 dpm(mmole)$^{-1}$ was isolated from the urine (51). The specific activity of urinary formate was approximately 10 % that of urinary acetate recovered from the urine after the administration of DL-[6-^{14}C]lysine and unlabelled acetate. The labelling pattern of glucose recovered from the urine after the administration of DL-[6-^{14}C]lysine and unlabelled glucose was similar to that obtained after the injection of [^{14}C]formate (51). Rothstein and Miller (51) interpreted the results of these experiments to indicate that carbon atom 6 of lysine might be directly converted to formate in a minor reaction of lysine. However, no other report of the formation of formate from lysine has been found. On the other hand, when L-[U-^{14}C]lysine or DL-[6-^{14}C]lysine was injected into rats every day for 4 days no significant radioactivity was found in the methyl groups of carnitine isolated from the carcasses after killing on the fifth day (13). Since the label from [Me-^{3}H]methionine was incorporated into the methyl groups of carnitine (13) and since [^{14}C]formate would be expected to label methionine in the methyl position, this result indicates that there was not significant conversion of lysine to formate in the rats.

Since a search of the literature has revealed no other metabolic fate of carbon atom 1 of lysine in animals, it appears that if carbon atom 1 is not incorporated unchanged into protein, it is oxidized to CO_2, with only a relatively small amount being incorporated into pipecolic acid in the rat.

Hutzler and Dancis (35) have investigated the tissue distribution of lysine-α-ketoglutarate reductase (no systematic designation), which catalyzes the formation of saccharopine from L-lysine and α-ketoglutarate. This reaction is the first step in the major pathway of lysine catabolism in animals. In humans this enzyme had the highest activity per g of tissue in liver, with kidney and heart having activities 23 % and 11 % of the liver (35). Other tissues had lower specific activities and only a trace of activity was found in skeletal muscle (35). The level of activity of lysine-α-ketoglutarate reductase was found to be similar in liver tissue from human, rat, pig, dog, cat, ox and sheep (16), indicating that the use of L-[1-^{14}C]lysine in the method for measuring protein metabolism may be applicable to a variety of animals.

More than 90 % of the CO_2 produced from carbon atom 1 of lysine is likely to be expired. The expiration of $^{14}CO_2$ following the intraperitoneal injection of [^{14}C]carbonate or [^{14}C]bicarbonate has been investigated using mice and rats; 94 to 98 % of the injected radioactivity was recovered in the expired air (3, 23, 24, 52, 53). The urine collected for 24 h after the injection of [^{14}C]carbonate was found to contain 0.5 to 3.7 % of the injected radioactivity (3, 24, 53), and after 24 h, tissues of mice were found to retain only 1.37 % of the injected radioactivity (52). The expiration of $^{14}CO_2$ occurred rapidly; 93 % of injected label was collected in 1 h (53) and 94 % within 2 h (52, 53).

Endogenously produced CO_2 might not be expired to the same extent as CO_2 produced from intraperitoneal injections of carbonate or bicarbonate. The fixation of $^{14}CO_2$ produced from the oxidation of L-[1-^{14}C]lysine may be experimentally determined by analysis of empty body mass for non-lysine radioactivity after the injection of L-[1-^{14}C]lysine. A correction could then be applied in the estimation of protein synthesis for the percent fixation of $^{14}CO_2$ generated from L-[1-^{14}C]lysine.

In the proposed method of estimating protein synthesis, accretion and degradation based on the metabolism of lysine, it would be necessary to determine the dietary absorption of lysine. Although in the present study phenylalanine absorption was estimated from total N absorption, the availability in the digestive tract of dietary lysine does not correlate well with the availability of dietary N (37). Therefore, the absorption of lysine ($fd^{\dagger}$)[1] probably should not be estimated from the total N availability of the diet. Amino acid analysis of the feed and feces has been used as an estimate of the availability of dietary amino acid (37), and may be an adequate

[1] Symbols analogous to those described in the Theory for the metabolism of phenylalanine are used here for the metabolism of lysine and distinguished from phenylalanine by a †.

means of determining a value for lysine absorption.

After the administration of L-[1-^{14}C]lysine, some L-[1-^{14}C]-lysine, [^{14}C]pipecolic acid, [^{14}C]urea and $^{14}CO_2$ would likely be excreted in the urine. In determining the proportion of administered L-[1-^{14}C]lysine incorporated into body protein the amount of radioactivity found in L-[1-^{14}C]lysine and [^{14}C]pipecolic acid in the urine should be subtracted from the administered dose. The remaining radioactivity found in the urine should be added to that recovered in the expired $^{14}CO_2$.[1] The amount of $^{14}CO_2$ found to be fixed in the tissues should also be added to that recovered in the expired $^{14}CO_2$. All of these corrections would likely be relatively small, and the same values could probably be applied under a variety of experimental conditions without introducing a significant error.

Except for the corrections just mentioned, any ^{14}C not recovered in the expired air after the injection of L-[1-^{14}C]lysine could be assumed to have been incorporated into protein as unchanged L-[1-^{14}C]-lysine. The corrected proportion of radioactivity recovered in the expired $^{14}CO_2$ after the administration of L-[1-^{14}C]lysine ($M^{\dagger}$) would be analogous to M in experiments already described using L-[U-^{14}C]-phenylalanine and L-[U-^{14}C]tyrosine. The value of $M^{\dagger}$ determined using L-[1-^{14}C]lysine, however, would be free of an error of the type resulting from the difference in metabolism of plasma L-[U-^{14}C]-tyrosine and L-[U-^{14}C]tyrosine derived endogenously from phenylalanine.

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[1] The proportion of radioactivity in the urine due to the excretion of L-[1-^{14}C]lysine and [^{14}C]pipecolic acid may be determined in a manner analogous to that used to estimate the urinary excretion of phenylalanine and derivatives of phenylalanine other than those formed from tyrosine.

b. The Use of Ad Libitum Feeding and Correcting for Diurnal Variation

Ad libitum feeding may provide more meaningful estimates of protein turnover in relation to practical conditions of existence of animals. For example, as mentioned in section B of the Discussion, Obled, Arnal and Fauconneau (47) found that rats receiving meals every 4 h had a considerably lower rate of protein synthesis for visceral proteins than rats fed ad libitum. With an ad libitum feeding regimen the measured values of both $fd^{\dagger}$ and $r^{\dagger}$ would be valid only on a daily basis, and therefore, should not be used to calculate protein synthesis from $M^{\dagger}$ determined at only one time of the day. A value of $M^{\dagger}$ which approximates the daily mean should be estimated.

Single injections of L-[1-^{14}C]lysine, followed by the total collection of expired $^{14}CO_2$, could be used to estimate $M^{\dagger}$. A collection period for expired $^{14}CO_2$ of 3 h or longer may be required. The results of an injection during the day and another during the night would probably reflect the extremes of diurnal differences in the oxidation of L-[1-^{14}C]lysine. The mean value of $M^{\dagger}$ calculated from the results of both injections may be acceptible to use in the calculation of protein synthesis.[1]

The error which could result from the use of a mean value of $M^{\dagger}$ would depend upon both the absolute and relative values of the individual measurements of $M^{\dagger}$. Larger values and a greater difference between the two individual values would result in a greater potential maximum error in the estimation of the rate of protein synthesis. Nevertheless, the rate of protein synthesis determined in this manner should be greater than the minimum rate during the day and less than the maximum, and should, therefore, approach the real daily value.

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[1] Prior to the second injection of L-[1-^{14}C]lysine a measurement of the residual background expiration of $^{14}CO_2$ should be obtained.

SUMMARY

A method for the estimation of protein turnover in rats has been developed and proposed in this thesis. Using rats on a continuous feeding regimen, rates of total protein synthesis, accretion and degradation have been estimated from studies of N balance and phenylalanine metabolism. Quantitative estimates of the catabolism of phenylalanine and its incorporation into protein were determined from the rates of oxidation of continuously infused L-[U-^{14}C]phenylalanine and L-[U-^{14}C]tyrosine. Protein synthesis was calculated from the rate of incorporation of phenylalanine into body protein according to a single-pool model of phenylalanine metabolism. Protein accretion was estimated from N balance, and protein degradation was calculated as the difference between synthesis and accretion.

The route of administration of L-[U-^{14}C]phenylalanine and L-[U-^{14}C]tyrosine did not significantly affect the estimation of protein turnover. Furthermore, the rate of protein synthesis in the whole rats appeared to be a function of the mass of the animal, whereas the rate of protein accretion appeared to be a function of dietary N consumption.

The estimates of protein synthesis and degradation obtained were considerably lower than the limited number of comparable estimates available in the literature. The low values may be attributed to the use of a restricted, continuous feeding regimen and to the difference in metabolism between endogenously synthesized L-[U-^{14}C]tyrosine and L-[U-^{14}C]tyrosine infused into the plasma. The proposed method may provide more accurate and meaningful results if an ad libitum

feeding regimen was used and if injections of L-[1-^{14}C]lysine were substituted for infusions of L-[U-^{14}C]phenylalanine and L[U-^{14}C]-tyrosine.

The method has been used with intact animals that need not be sacrificed at the end of the experiment. Since all measurements may be performed on each animal, large numbers of animals are not needed, and an indication of the variation in protein metabolism between animals may be obtained. The method is not difficult, nor should it be time consuming or expensive, especially if the suggested modifications are employed.

It is felt that the present investigations have contributed to the development of a method for the estimation of protein synthesis, accretion and degradation in whole animals. In particular, the results obtained have demonstrated the advantages and disadvantages of the proposed method, and have, therefore, indicated by what means the method may be improved and used for practical measurements of animal protein metabolism.

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APPENDIX A

Automatic Feeder

An automatic feeder was designed to feed eight rats in individual metabolism cages. The animals were fed from individual feed hoppers, which were removed and weighed each day. Daily weighing of the hoppers provided a precise determination of the amount of feed dispensed to each rat. The hoppers were refilled each day with standard rat diet ground to pass a screen of about 16 mesh. Feed was lifted from the hoppers by cups attached to discs on a central shaft. With each revolution of the shaft one aliquot of feed was unloaded into a funnel mounted inside each hopper. These funnels directed the feed into polyethylene tubes, which in turn directed the feed to the metabolism cages. In order to minimize waste and spillage, the feed was dispensed into confinement feeders. The frequency with which the feed was dispensed was regulated by a recycling timer (Dual-Trol Recycling Timer, model TM-3H; Vapor Canada). By varying the frequency of distribution or the size of the cups the machine could be adjusted to the requirements of the feeding regimen. In the experiments presented in this thesis, the rats were fed about 0.27 g of feed twice per hour. Once the rats were accustomed to the feeding regimen, observations indicated that they consistently ate the feed as soon as it was presented.

Photographs of the automatic feeder and its components are provided in Plates 1 to 5. A description of the electrical circuit for the feeder is provided in Fig. 4.

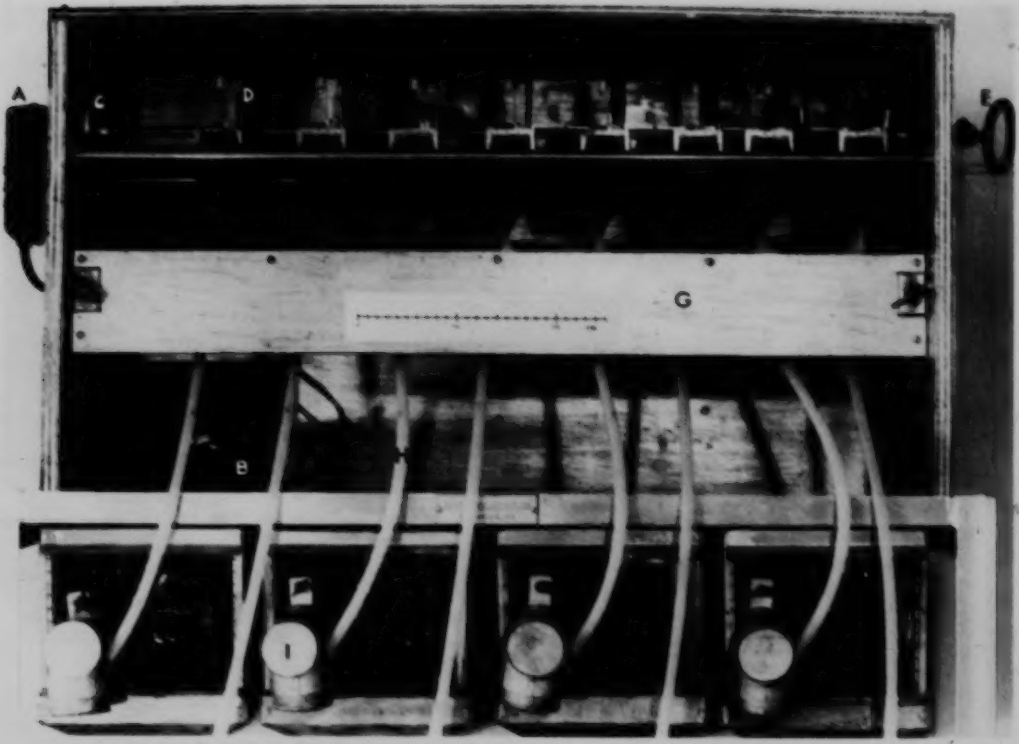

Plate 1. Automatic feeder mounted on a battery of rat metabolism cages. The feeder was driven by a barbecue spit motor (A). When activated by the timer (B), the motor turned a central shaft, on which were mounted a cam (C), 8 discs for dispensing feed (D) and a counter balance (E). Eight stainless steel feed hoppers (F) were mounted on bars beneath the central shaft. A shelf (G) supported polyethylene funnels used to catch feed dispensed from the hoppers. Polyethylene tubes (9 mm ID) (H) were attached to the funnels, and directed the feed into confinement feeders (I) on the rat metabolism cages. The tubes were fitted into holes drilled into the sides of the confinement feeders. The shelf (G) rotated back on a separate bar allowing easy access to the feed hoppers.

2

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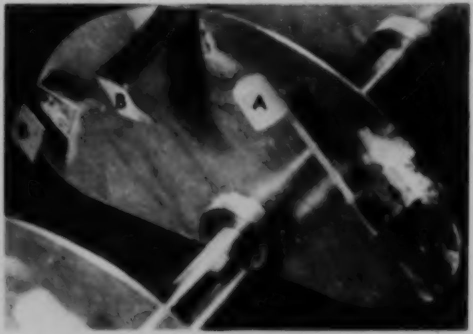

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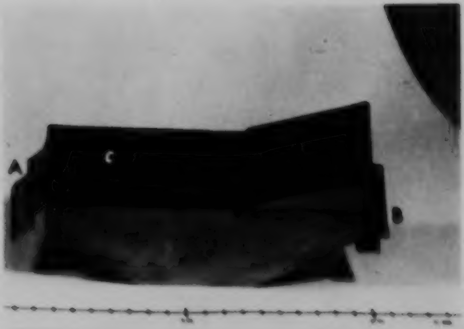


5

Plate 2. Cam (A) and microswitch (B). The diameter of the cam is 12 cm. As shown, the microswitch is in line with a depression in the cam, and as such is in the normally open position. Plate 3. Feeding disc, with cup (A) for dispensing feed, and vanes (B) for mixing feed in the hopper. The interapical distance across the disc is 15 cm. Plate 4. Feed hopper. Note the spring clip (A) and the bracket (B) used for mounting the hopper in the automatic feeder. The funnel (C) is fitted inside and empties through the bottom of the hopper. Plate 5. Feed discs and hoppers mounted in the automatic feeder. The hoppers are held in position by means of the brackets and spring clips (see Plate 4). The hoppers, which had a tare weight of about 370 g, were readily removed for weighing and refilling. The amount of feed dispensed each day was determined by weighing the hoppers at the beginning and end of the 24 h period. When the feed discs were turned one revolution, one cup (approximately 0.27 g) of feed was passed to the polyethylene funnels (A) beneath the hoppers and dispensed to the individual rats in the metabolism cages.

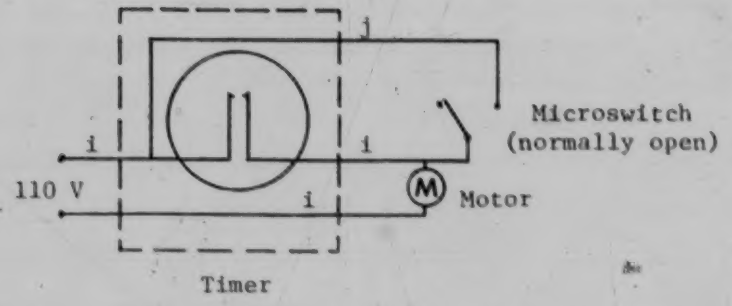


Fig. 4. Circuit diagram for automatic feeder. The recycling timer was set to close circuit i for 15 sec every 30 min. During the 15 sec the motor (shaded pole barbecue spit motor with fractional horse power) drove a shaft turning the feed cup discs and a cam. The microswitch was closed by the cam and remained closed for nearly a complete revolution. While the microswitch was closed, circuit j was also closed so that the motor continued running after circuit i was opened by the timer. At the end of one revolution, the microswitch was opened by the cam causing the motor to stop until the timer once again activated the circuit.

APPENDIX B

Catheterization of Tail Vein

A tail vein was catheterized just prior to the beginning of each infusion. The catheter consisted of the stem of a 27 gauge needle attached to a section of 0.28 mm ID polyethylene tubing (Intramedic, PE10; Clay Adams). The needle was inserted in the cranial direction into a tail vein and its position checked by injecting an autoclaved solution of lisamine green in physiological saline. When the needle was correctly positioned the green dye could be seen travelling up the vein. If incorrectly positioned, a green blister would form. Once correctly positioned, the needle was held in place with a piece of tape, and the polyethylene tubing was attached to the infusion pump. Catheterization normally took about a minute, with apparently little discomfort to the rat.

Aseptic procedures were followed during catheterization, as well as during the preparation of the infusate and infusion. Equipment which was not autoclaved was treated with a 0.1 % solution of alkyldimethylbenzylammonium chloride (Zephiran).

APPENDIX C

Collection and Counting of Expired $^{14}CO_2$

Expired $^{14}CO_2$ was collected by scrubbing through a scintillation fluid of the following composition:

500 ml toluene

300 ml ethylene glycol monomethyl ether (Methyl Cellosolve; Union Carbide Canada)

200 ml monoethanolamine

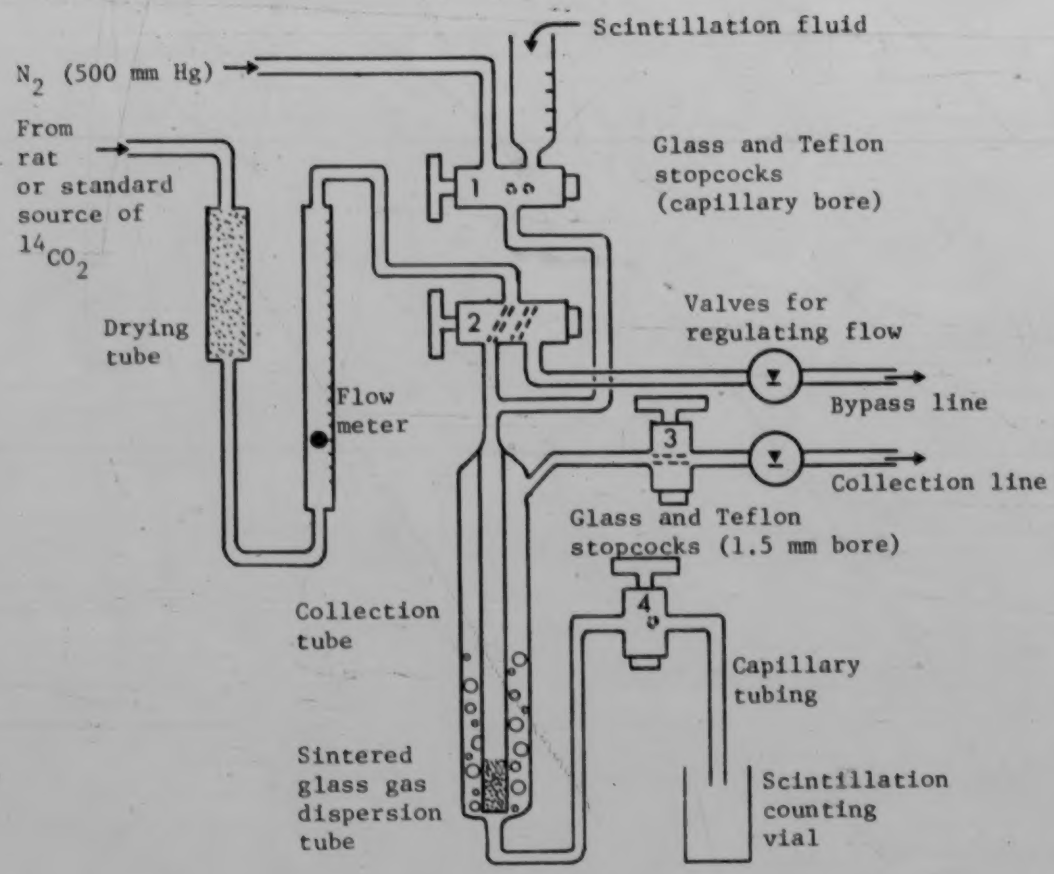
5.0 g 2,5-diphenyloxazole (PPO)

0.20 g 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP)

A trace of Antifoam A (Dow Corning Corp.) was used to control foaming.

The toluene and scintillators (PPO and POPOP) were mixed well in advance, but the other components were added within 2 days of using the scintillation fluid. Foaming during collection was unpredictable if this precaution was not taken.

Air was drawn past the rat and through the collection system (Fig. 5) by negative pressure. Whether the flow was directed through the scintillation fluid or through the bypass, it was regulated at approximately 1.0 $l(min)^{-1}$ by the use of valves (no. 1RS4; Whitey Research Tool Co.) and a flow meter (size 2; Roger Gilmont Instruments). Thus, air flow past the rat remained constant even though $^{14}CO_2$ was collected for only 30 min of each hour. Before bubbling through the scintillation fluid, the air was dried with indicating Silica Gel (3 to 8 mesh; Canadian Laboratory Supplies).



Scintillation fluid
N_2 (500 mm Hg)
From rat or standard source of $^{14}CO_2$
Glass and Teflon stopcocks (capillary bore)
1
2
Drying tube
Flow meter
Valves for regulating flow
Bypass line
3
Collection line
Glass and Teflon stopcocks (1.5 mm bore)
4
Collection tube
Capillary tubing
Sintered glass gas dispersion tube
Scintillation counting vial

After a 30 min collection period, the scintillation fluid was decanted directly into a scintillation vial and the collection apparatus was washed with two 4 ml aliquots of scintillation fluid, which were also collected in the same vial. In order to remove the last of the radioactivity from the collection tube, another 8 ml and two more 4 ml aliquots were washed through the apparatus and collected in a second vial. About 95 % of the recovered radioactivity was found in the first vial. The radioactivity collected was counted at an efficiency of about 70 %.

SECTION II

CHARACTERISTICS
OF
IN VITRO INACTIVATION
OF
TYROSINE AMINOTRANSFERASE

INTRODUCTION

In recent years the number of papers published on protein turnover has reflected a developing interest in this area of metabolism. Defined as the flux of amino acids through protein (43), protein turnover is a process involving virtually all of the proteins within mammalian cells (28). Although ubiquitous, it is not well understood. In particular, the mechanism of intracellular protein degradation will undoubtedly require a great deal of effort to elucidate. In the experiments presented here, the inactivation of tyrosine aminotransferase (L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.5) in rat liver homogenates has been used as a model for the investigation of characteristics of protein degradation.

Although the mechanism of intracellular protein degradation is poorly understood, it has become apparent that complex, strictly controlled reactions are involved. However, the lack of a cell free system from mammalian tissues with which to study the mechanism has hampered investigations. Recently, the inactivation and degradation of tyrosine aminotransferase was observed by Auricchio, Mollica and Liguori (3) in specially prepared rat liver homogenates. In an attempt to establish a cell free system for the study of protein degradation it was decided to confirm and, if possible, extend those observations.

It has been noted that tyrosine aminotransferase degradation in rat liver may provide a particularly suitable model system for the study of protein degradation (38). Information available about the characteristics of tyrosine aminotransferase degradation indicates that reactions common to general protein degradation might be involved. Thus, it was hoped that a study of tyrosine aminotransferase may contribute to an understanding of the general mechanism.

LITERATURE REVIEW

A. Heterogeneity and Specificity of Degradation Rates

There is a marked heterogeneity in the rates of degradation of different proteins in tissues (28, 91, 97). Most studies of turnover rate have been made with liver proteins, although observations with other tissues (18, 28, 49, 91) have indicated that multiplicity of turnover rates amoung proteins is a general characteristic. Estimates of the half-life of tyrosine aminotransferase have ranged from 1.5 to 5 h (91) in rat liver, while the half-lives of nearly 40 other enzymes have been found to vary from 11 min for ornithine decarboxylase (L-ornithine carboxy-lyase, EC 4.1.1.17) to 16 days for isozyme 5 of lactate dehydrogenase (L-lactate:NAD$^+$ oxidoreductase, EC 1.1.1.27) (28).

The double isotope technique described by Arias, Doyle and Schimke (2) and critically evaluated by Dice, Dehlinger and Schimke (18) and Goldberg (28) has been successfully used to determine the relative degradation rates of a great variety of liver proteins. Those studied included soluble (13, 18, 23, 34), mitochondrial (14, 34), ribosomal (14, 19), chromosomal (20) and microsomal (2, 14) proteins. Electrophoretic or chromatographic separation of the proteins of each cell fraction indicated that all proteins separated were degraded at characteristic rates in vivo, including particle and membrane bound proteins. It is apparent, therefore, that the mechanism of intracellular protein catabolism must provide for a specific rate of degradation for each protein.

B. Random Degradation

Since lifetime kinetics have been found only for hemoglobin (96), while virtually all enzymes have been observed to be degraded according to first order kinetics (98), Schimke (97, 98) has suggested that degradation is a random process. In other words, except for hemoglobin, all molecules of a protein species appear to have an equal probability of being degraded, regardless of when they were synthesized.

C. Intrinsic Characteristics of Proteins which Could Influence Their Degradation

Certain characteristics of protein molecules may act as determinants or rate limiting factors for their own degradation. If the specificity required to give each protein a characteristic rate of degradation was a function of the protein being degraded, then the catabolic system itself could be ~~relatively~~ nonspecific.

a. Molecular Weight

Studies using the double isotope technique of Arias, Doyle and Schimke (2) have revealed that proteins with larger molecular weights tend to be degraded in vivo more rapidly than proteins with smaller molecular weights (13, 14, 18, 19, 20, 23, 34).

The same correlation has not been found for tyrosine aminotransferase (mol. wt. 115,000 [118]), tryptophan 2,3-dioxygenase (L-tryptophan:oxygen 2,3-oxidoreductase [decyclizing], EC 1.13.11.11; mol. wt. 167,000 [102]) and arginase (L-arginine amidinohydrolase, EC 3.5.3.1; mol. wt. 118,000 [41]), which have half-lives of 1.5 to 5 h, 2.3 to 4 h and 4 to 5 days, respectively (91). However, an extensive correlation between molecular weights and half-lives of enzymes has not been made.

Tweto, Dehlinger and Larrabee (112) have reported that the larger subunits of the multienzyme complex of rat liver fatty acid synthetase were degraded with shorter half-lives than the smaller subunits. Dice and Schimke (13) found that the subunits of rat liver soluble proteins demonstrated a negative correlation between molecular weights and half-lives. These results have led to the suggestion that proteins are degraded after dissociation into subunits (14, 19, 20, 23).

b. Conformation and Non-native Structures

Proteins containing abnormal amino acid sequences resulting from the incorporation of an amino acid analogue or a genetic defect during synthesis may be expected to possess a conformation different from that of the native protein. Goldberg (26) and Pine (86) demonstrated that bacterial proteins containing defects of this sort were rapidly and selectively degraded in vivo. Similarly, abnormal hemoglobin was rapidly degraded within reticulocytes (1, 90). Thus, one factor which could influence the in vivo rate of degradation of proteins may be the presence of abnormal structures.

Studies with tyrosine aminotransferase have not been consistent with this hypothesis. The presence of amino acid analogues during the induction of tyrosine aminotransferase in cultured hepatoma cells produced an enzyme that was less heat stable than the native enzyme (48). However, both the analogue-containing enzyme and the native enzyme were degraded with the same half-life in the cell culture (48).

c. Ligands

Numerous examples of substrates, cofactors or other ligands affecting the stability of proteins in vivo and in vitro have led to the development of a widely accepted model of protein degradation (28, 96). In this model protein molecules were described as being individually available to a degradative system considered to be present in excess. Since protein molecules may exist in a number of states and a thermodynamic equilibrium is expected to exist between these states, ligands may exert control over the state of the protein molecule by influencing the equilibrium. In some state or states the protein molecule is considered to be vulnerable to degradation or inactivation, whereas in other states it is stable.

This model was derived largely from observations of the stabilizing effects of ligands on enzymes. For example, tryptophan decreased the rate of degradation of tryptophan 2,3-dioxygenase in vivo (57, 99), and pyridoxal 5'-phosphate and α-ketoglutarate stabilized tyrosine aminotransferase against heat in vitro (16). Litwack and Rosenfield (63) have suggested that for the rapidly turning over enzymes of rat liver, the dissociation of coenzymes from holoenzymes may be the rate limiting step in degradation.

d. Correlation Between In Vivo Rate of Degradation and In Vitro Susceptibility to Proteinases

It is generally assumed that the process of intracellular protein degradation must ultimately involve proteolytic hydrolysis. Accordingly a number of studies have been performed to investigate the relationship between the in vivo rate of degradation of different proteins and their susceptibility to well characterized proteolytic enzymes. The results have indicated that the in vivo half-lives of many proteins could be estimated from the rates at which they are attacked by proteolytic enzymes.

Goldberg (27) has recently demonstrated that both normal and abnormal proteins of *Escherichia coli* were degraded by trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1), subtilisin (EC 3.4.21.14) or Pronase (no systematic designation) at rates reflecting in vivo catabolism. Within a mixture of chromosomal proteins extracted from rat liver chromatin with 2 M NaCl those proteins more rapidly degraded in vivo were also more rapidly degraded by Pronase (14). Similar results were found for soluble rat liver proteins (18). When incubated with Pronase or subtilisin, which are less specific proteinases than trypsin or chymotrypsin, the rates of degradation of lactate dehydrogenase and tyrosine aminotransferase did not correlate well with in vivo rates of degradation (4). The rates of degradation of arginase, catalase (hydrogen-peroxide:hydrogen-peroxide oxidoreductase, EC 1.11.1.6) and serine dehydratase (L-serine hydro-lyase [deaminating], EC 4.2.1.13), though, correlated with in vivo rates in the same study (4). Although Schimke, Sweeney and Berlin (100) found that tyrosine aminotransferase was stable in the presence of trypsin, Bond (4) found that the proteolytic susceptibility of rat liver tyrosine aminotransferase as well as arginase, lactate dehydrogenase, catalase and serine dehydrogenase correlated well with in vivo catabolic rates when incubated with trypsin or chymotrypsin.

It seems probable that any proteolytic enzymes involved in intracellular protein degradation would have characteristics different from those of trypsin, chymotrypsin, subtilisin and Pronase. The anomalous stability of lactate dehydrogenase and tyrosine aminotransferase in the presence of Pronase and subtilisin could be due to such variations in proteolytic specificity. In fact, it has recently been shown that tyrosine aminotransferase was rapidly inactivated by lysosomal proteinases from rat liver (3).

The extreme situation of a specific intracellular proteinase for each type of protein molecule is logically impossible as pointed out by Schimke (96). It appears possible, though, that a few relatively nonspecific proteinases could catalyze the in vivo degradation of proteins. A mechanism of this sort could be regulated by factors such as molecular weight and conformation of the protein being degraded and the presence of certain ligands.

D. Intracellular Proteolytic Enzymes

Proteolytic enzymes which could be involved in the intracellular mechanism of protein catabolism are found in a variety of tissues and cell fractions. Those most extensively studied are the cathepsins found in lysosomes. The pH optimum of cathepsins is in the acid range with little activity observed at pH 7. At pH 4.4 lysosomal extracts have been shown to catalyze the extensive degradation of a number of proteins, with the production of ninhydrin positive material equal to 70 % of that obtained by acid hydrolysis (11). In addition to free amino acids, the products of enzymatic digestion included small peptides (mostly dipeptides) (11). The mixture of small peptides was shown to be further hydrolyzed by rat liver soluble fraction at pH 8.0 (11), indicating that liver cells possess all of the enzymes required for the complete hydrolysis of proteins to amino acids.

Because lysosomes contain proteolytic enzymes it has frequently been suggested that these particles may serve an important function in the catabolism of intracellular proteins (11, 12, 96, 97, 98). Evidence that lysosomes are a site of intracellular proteolysis has recently been obtained by Mortimore and co-workers (72, 74). Amino acid production was studied in unbuffered homogenates prepared from perfused rat livers subjected to treatments that influence protein degradation. A significant production of amino acids and peptides by the total particulate fraction was observed after perfusion with or without glucagon, whereas perfusion with amino acids or insulin inhibited the apparent proteolytic activity (72). In other studies (74) liver protein was labelled in vivo by intraperitoneal injections of L-[1-^{14}C]leucine 18 and 4 h prior to perfusion. Differential centrifugation of liver homogenates prepared after perfusion revealed that trichloroacetic acid soluble radioactivity was associated mainly with the lysosomal fraction. Since mitochondria and lysosomes prepared from unlabelled livers did not absorb radioactivity from the supernatant fractions, the results indicated that lysosomes were a site of proteolytic degradation in the perfused livers.

Particle bound catheptic activity and alkaline proteolytic activity bound to the myofibrillar fraction have been found in skeletal muscle (75, 76, 77, 79, 80, 109). Starvation or protein deficiency in growing rats produced an increase in both the acid and alkaline proteinase activity (78), and it has been shown that protein and calorie deficiencies resulted in increases in the rate of myofibrillar protein catabolism in vivo (69). Furthermore, alkaline proteinase activity increased after glucocorticoid administration (65), which correlates with a demonstration that cortisone atrophy of skeletal muscle was accompanied by increased protein catabolism (24). Under conditions of muscle wasting such as vitamin E deficiency and hereditary muscular dystrophy, increases in muscle catheptic activity have been observed (47, 79, 122). The fluctuations in both acidic and alkaline proteolytic activity corresponding to certain physiological states suggests that these enzymes may contribute to degradation of protein in skeletal muscle.

Katunuma and co-workers (49, 50, 53, 58) have recently reported the presence of selective proteinases in skeletal muscle and small intestine. Extremely low levels of activity were also found in brain, heart, spleen, large intestine, stomach, kidney and liver (52, 58). These enzymes have been called "group-specific proteinases". Two types have been found; those which demonstrated relative specificity for the apoenzyme of NAD-dependent dehydrogenases and those which demonstrated relative specificity for the apoenzyme of pyridoxal-dependent enzymes. The latter has been purified 500 fold from the small intestine and has been more intensively studied than the former. In response to niacin or vitamin B_6 deficiency, the specific activities for inactivation of NAD-dependent dehydrogenases or pyridoxal-dependent enzymes, respectively, were increased (51, 52). The enzyme inactivating pyridoxal-dependent enzymes was found to inactivate ornithine-oxo-acid aminotransferase (L-ornithine:2-oxo-acid aminotransferase, EC 2.6.1.13) apoenzyme by splitting the apoenzyme into two products, one of which still possessed antigenic activity with the antibody for ornithine-oxo-acid aminotransferase (58). This fragment was rapidly degraded by trypsin while ornithine-oxo-acid aminotransferase apoenzyme was not (58). Tyrosine aminotransferase was only slightly inactivated by the small intestine inactivating enzyme (58), and not at all by the liver enzyme (49), while other pyridoxal-dependent enzymes were more extensively affected. These observations indicate that group-specific proteinases may serve an important function in the intracellular regulation of certain enzymes, but tyrosine aminotransferase may not be one of them.

E. *Other Factors Regulating Intracellular Protein Degradation*

Although proteinases and peptidases are probably required for the hydrolysis of proteins to amino acids, other observations have shown that the regulation of intracellular protein degradation involves additional factors.

a. Energy

The early studies of Simpson (105) and Steinberg and Vaughan (111) demonstrated an inhibition of the degradation of proteins in rat liver and kidney slices under conditions (anaerobiosis, cyanide, 2,4-dinitrophenol) that inhibited respiration and oxidative phosphorylation. Later, Schimke, Sweeney and Berlin (100) as well as Brostrom and Jeffay (6) confirmed these results. Mandelstam (64) found that protein degradation in *Escherichia coli* was almost completely inhibited by 2,4-dinitrophenol and by azide within 3 h after addition to non-growing cells. More recent evidence (26, 87, 89, 104) has also indicated that protein degradation in *Escherichia coli* is an energy dependent process. The function of respiration and oxidative phosphorylation in protein degradation, however, is unclear.

The inactivation of tyrosine aminotransferase and tryptophan 2,3-dioxygenase in liver slices correlated well with the observed effect of anaerobiosis on general protein degradation. In liver slices, tryptophan 2,3-dioxygenase enzymatic activity and antigenic activity as well as tyrosine aminotransferase enzymatic activity were rapidly lost under aerobic conditions (62, 100), but were stable under anaerobic conditions (100).

There are a number of reports in the literature in which it is claimed that ATP is required for intracellular protein degradation. Many of these reports, however, have been discounted by later work. It was concluded by Umaña (113, 114, 115, 116) and Umaña and Feldman (117) that protein degradation observed in neutral rat liver homogenates was an ATP dependent process. However, it has been adequately shown by Hunter and Harper (46) and by Goldspink and Goldberg (29) that this conclusion was due to experimental error. Similarly, Hayashi, Hiroi and Natori (36) presented evidence that ATP stimulated protein catabolism by rat liver lysosomal preparation below pH 5. However, the investigations of Huisman, Bouma and Gruber (44, 45) indicated that facilitation of lysosome disruption by ATP at pH 4.5 was responsible for the observation.

Recent results reported by Hershko and Tomkins (38) indicated that the degradation of tyrosine aminotransferase in hepatoma cell culture was dependent on ATP. Following induction with dexamethasone, tyrosine aminotransferase activity usually returned rapidly to the preinduction level. However, the addition of NaF or KF profoundly inhibited the degradation of both induced tyrosine aminotransferase and general cell proteins. The activity of the enzyme was shown to correlate with enzyme protein measured by a specific radioimmunoprecipitation method. Furthermore, in the absence of glucose, however, the addition of 2,4-dinitrophenol or sodium azide did not inhibit degradation. Concomitant measurements of intracellular ATP levels fully agreed with the interpretation of a requirement for ATP for enzyme degradation. Whether the role of ATP in enzyme degradation was direct or indirect was not determined, but it was proposed that ATP participates in an early phase of protein catabolism since it was required for the loss of enzyme activity.

These results, though promising, should be interpreted with caution. Although the incidence of low ATP levels correlated well with inhibition of enzyme degradation, the inhibition could have been due to other factors resulting from the disruption of cellular metabolism. Peterkofsky (83) has observed that both acid phosphatase (orthophosphoric-monoester phosphohydrolase [acid optimum], EC 3.1.3.2) and alkaline phosphatase (orthophosphoric-monoester phosphohydrolase [alkaline optimum], EC 3.1.3.1) caused an apparent loss of tyrosine aminotransferase activity in vitro, which may have been due to the dephosphorylation of pyridoxal 5'-phosphate. NaF inhibited acid phosphatase activity and the apparent inactivation of tyrosine aminotransferase. Although Peterkofsky observed that the addition of pyridoxal 5'-phosphate to the enzyme assay medium overcame the inactivation caused by acid phosphatase, the intracellular concentration of pyridoxal 5'-phosphate might be expected to exert a controlling effect on tyrosine aminotransferase degradation. Thus, in the experiments of Hershko and Tomkins (38), tyrosine aminotransferase may have been stabilized by a high level of pyridoxal 5'-phosphate as a result of inhibition of acid phosphatase by NaF rather than a low level of ATP.

Mego and co-workers (66, 67) have found that ATP may be required for the maintenance of an acid pH within lysosomes. Mouse liver heterolysosomes prepared from animals previously injected with denatured [^{131}I]albumin released trichloroacetic acid soluble radioactivity when incubated at pH 5 (66). Release of trichloroacetic acid soluble radioactivity was much slower at pH 7, but was stimulated by the addition of ATP (67). ATP appeared to prevent heterolysosome breakage and to promote proteolytic activity. It was suggested (67) that ATP may provide the necessary energy to drive a proton pump in the heterolysosome membrane and thus maintain a low pH within the particles. As a result, catheptic activity could proceed at or near its optimum pH.

Although some results have been obtained which indicate that intracellular protein degradation requires ATP, it is clear that further work will be necessary in order to explain the requirement for respiration and oxidative phosphorylation originally observed by Simpson (105) and Steinberg and Vaughan (111).

b. *Tissue Structure*

Several studies have indicated that the structural integrity of tissue or at least cell organelles is required for protein degradation and enzyme inactivation to proceed. Studies with perfused livers (62, 103), liver slices (63, 100) and liver homogenates (62, 100) indicate that the inactivation of tryptophan 2,3-dioxygenase and tyrosine aminotransferase may require intact liver cells or cellular structures. In perfused livers, following glucocorticoid induction, tryptophan 2,3-dioxygenase enzymatic and antigenic activity decreased at a rate comparable to in vivo degradation (62). Tyrosine aminotransferase, on the other hand, showed an anomalous increase in activity under these conditions (62). In liver slices, tryptophan 2,3-dioxygenase enzymatic activity and antigenic activity as well as tyrosine aminotransferase enzymatic activity were rapidly lost under aerobic conditions (62, 100). In aerobic homogenates, however, tyrosine aminotransferase activity was stable (100), while tryptophan 2,3-dioxygenase rapidly lost activity (62, 100). The apparently normal degradation of protein, which occurred in perfused livers and liver slice preparations, was not observed in rat liver homogenates under any conditions (6, 62, 100). Thus, tryptophan 2,3-dioxygenase inactivation correlated with general protein degradation in perfused livers and liver slices, whereas tyrosine aminotransferase inactivation correlated with general protein degradation in slices and homogenates.

Loss of tryptophan 2,3-dioxygenase antigenic activity in aerobic homogenates was observed by Li and Knox (62), but not by Schimke, Sweeney and Berlin (100). Inactivation of purified, labelled tryptophan 2,3-dioxygenase did not result in the production of trichloroacetic acid soluble radioactivity in homogenates, nor was the addition of homogenate necessary for inactivation of the purified enzyme in the presence of O_2 (62). Nevertheless, it was concluded by Li and Knox (62) that the inactivation of tryptophan 2,3-dioxygenase observed in aerobic homogenates may have been consistent with in vivo degradation. It was concluded by Schimke, Sweeney and Berlin (100), however, that degradation of tryptophan 2,3-dioxygenase and tyrosine aminotransferase occurred only in the metabolically and structurally intact tissue. In a series of experiments investigating the progressive disruption of liver tissue, Brostrom and Jeffay (6) also concluded that structurally intact tissue was required for general protein degradation to proceed.

Recently, Auricchio, Mollica and Liguori (3) have suggested that the homogenization procedures used by previous workers were too drastic and resulted in the disruption of cell organelles and the consequent inhibition of degradation. These workers have reported the inactivation of tyrosine aminotransferase in neutral rat liver homogenates. They found, however, that it was necessary to homogenize "very gently" in order for inactivation to occur. Centrifuging at 13,000 × *g* for 10 min or vigorous homogenization resulted in no inactivation. The rate of inactivation was accelerated 4 fold by the addition of cysteine, whereas inactivation was inhibited by the addition of inhibitors of cathepsins B and B_1. It was concluded that the activity of cathepsins B and B_1 and the presence and integrity of subcellular particles, probably lysosomes, were required for inactivation to occur.

c. Hormones and Inhibitors of Protein Synthesis

Although there are several contradictory reports (21, 86), it has usually been found that mammalian (38, 111, 121) and bacterial (22, 25, 64, 73, 93, 101, 120) degradation of general cell proteins was inhibited by the presence of inhibitors of protein synthesis. These observations suggest that there may be a factor associated with the synthesis of protein which also has a regulatory effect on degradation. The investigations of Goldberg (25) have indicated that aminoacyl-tRNA, an intermediate in protein synthesis, may inhibit protein degradation when present in excess. In *Escherichia coli* an excess of aminoacyl-tRNA inhibited protein catabolism, while a lack of aminoacyl-tRNA stimulated protein catabolism (25). Moreover, the inhibition of protein catabolism by chloramphenicol was prevented under conditions in which the synthesis of a full complement of tRNA was inhibited (25). These results suggested that an accumulation of aminoacyl-tRNA was the reason for the inhibition of protein degradation by inhibitors of protein synthesis.

Conditions which are conducive to the uptake of amino acids by liver tissue and to the synthesis of liver protein tend to inhibit protein degradation and tyrosine aminotransferase inactivation. Insulin (71) and amino acids (121) have been shown to inhibit general protein degradation in isolated perfused rat livers previously labelled with L-[1-^{14}C]valine. Insulin is known to stimulate amino acid uptake into liver proteins (59, 60, 81) and into isolated perfused livers (68). In experiments using liver slices (3), the addition of a mixture of amino acids and glucose probably resulted in the incorporation of amino acids by the liver slices. The inactivation of tyrosine aminotransferase was inhibited by this treatment. Since an increased incorporation of amino acids into liver free amino acid pools and into liver protein likely reflects an increased formation of amino acyl-tRNA, the inhibitory effect of adding amino acids may be due to an increase in intracellular concentration of aminoacyl-tRNA.

The effect of protein synthesis inhibitors on the degradation of tyrosine aminotransferase seems to depend on whether or not the enzyme has been hormonally induced, and if so, on the stage of the induction cycle of the enzyme at the time of administration of inhibitor. Kenney (56) found by a label and chase procedure that the half-life of tyrosine aminotransferase when not induced (basal level of activity) was about 1.5 h. The in vivo administration of cycloheximide or puromycin sufficient to inhibit enzyme synthesis resulted in a stabilization of the basal enzyme level over a 5 h period (56). Kenney concluded that tyrosine aminotransferase degradation as well as synthesis must be blocked by cycloheximide and puromycin.

Tyrosine aminotransferase activity can be induced in rat liver in response to cortisol. Peak activity occurs about 6 h after administration of the hormone. Enzyme activity then rapidly subsides with a half-life of about 1.7 h (32). When puromycin was administered during the initial stage of induction further induction was inhibited and the enzyme activity was stabilized for 6 h (33). If puromycin was administered during the period of peak induction there was a further increase in enzyme activity (33). When puromycin was administered during the inactivation phase there was also an increase in enzyme activity (31, 33). Radioisotope studies showed that this increase was not due to de novo enzyme synthesis (31), nor did it occur when cycloheximide was administered instead of puromycin (31). The administration of cycloheximide during the degradative phase of the induction cycle, however, resulted in inhibition of further inactivation (31). If puromycin was administered 10 h after the peak of induction when enzyme activity had almost returned to the basal level there was no apparent effect.

The difference between the effects of puromycin and cycloheximide suggested that puromycin may have an effect additional to the inhibition of protein synthesis. The apparent recovery of enzyme activity after partial inactivation strongly suggests that tyrosine aminotransferase inactivation proceeded by more than one step and that the first step was reversed by puromycin. In view of this discovery, it seems unlikely that the first step in the inactivation of tyrosine aminotransferase would be proteolytic attack on the enzyme molecule. The effect of puromycin on the recovery of enzyme activity may become an important tool in the study of the mechanism of inactivation.

F. *Summary*

This review of intracellular protein degradation has emphasized results obtained in studies of tyrosine aminotransferase. The degradation of tyrosine aminotransferase in liver tissue appears to be a multistep process. There may be at least one reversible initial step which can be inhibited by inhibitors of protein synthesis.

The initial step(s) may require ATP, and may also be regulated by pyridoxal 5'-phosphate, the substrates and characteristics of the enzyme molecule itself. Structurally intact tissue or at least cell organelles appear to be required, and the final degradation of the enzyme molecule likely involves intracellular proteolytic enzymes. In general, the loss of tyrosine aminotransferase activity from liver tissue has many of the characteristics of cellular protein degradation, which suggests that these processes share common reactions.

MATERIALS AND METHODS

Tyrosine aminotransferase was induced in rat livers by intraperitoneal injections of triamcinolone (9α-fluoro-16α-hydroxyprednisolone diacetate) (Sigma Chemical Co.). Ten to 20 h after injection the animals were killed and the livers were immediately removed. After preparation of liver homogenates under a variety of conditions, the change in tyrosine aminotransferase activity was measured during incubation of the homogenates.

An automated method for measuring tyrosine aminotransferase activity was developed in order to provide a continuous estimate of enzyme activity during incubation.

A. *Experimental Animals*

Male, Sprague-Dawley rats (80 to 145 g) were used for all of the experiments. Most of the experimental animals were obtained from the rat colony maintained by the Department of Animal Science, The University of Alberta. When suitable animals were not available from this colony they were obtained from Bioscience Animal Services, The University of Alberta. All animals had free access to standard laboratory rat diets and water up to the time of killing.

Rats were each given an intraperitoneal injection (1 to 2 ml) of a saline suspension of triamcinolone at a dose of 15 or 30 mg(100 g body weight)$^{-1}$ at 10, 11, 16 or 20 h before killing. Immediately after killing by decapitation, the livers were removed and washed in cold tap water and placed into ice-cold saline unless specified otherwise.

B. Buffers

A number of different buffers were used for the preparation of rat liver homogenates: a, Krebs-Ringer-phosphate (15); b, 0.017 M tris(hydroxymethyl)aminomethane (tris) and 0.137 M KCl; c, 0.030 M tris and 0.124 M KCl; d, 0.080 M tris and 0.074 M KCl; e, 0.308 M tris-maleate, prepared by mixing 0.308 M tris and 0.308 M maleic acid in the correct proportion to give the desired pH; f, 0.308 M tris-citrate, prepared in an analogous manner to buffer e; g, 0.100 M tris-citrate and 0.104 M KCl, prepared from a 3.08 × dilution of buffer f. These buffers have been referred to by letter and partial descriptions throughout this section of the thesis.

The pH of the buffers was adjusted with concentrated HCl, 10 N KOH, 0.308 M maleic acid, 0.308 M citric acid or 0.308 M tris where appropriate. Allowances were made for temperature and the buffering effect of liver tissue so that pH adjustment after homogenate preparation would be minimized.

Any buffered solutions containing cysteine, cystine, $CuSO_4$ or $Na_4P_2O_7$ were prepared with the appropriate buffer immediately before use. The pH was adjusted at this time if necessary.

C. Preparation of Liver Homogenates

The initial step in the preparation of liver homogenates was to macerate the tissue with scissors in a small volume of buffer. The pieces were then extruded through a disposable plastic syringe into a 10 ml capacity Potter Elvehjem tissue grinder, and homogenized with a Teflon pestle driven by a 0.63 cm (0.25 in) hand drill. A total of 10 volumes (ml[g liver]$^{-1}$) of buffer were used to prepare a homogenate.

The Teflon pestle was machined such that it was 0.21 mm less in diameter than a normal tight fitting pestle in the Potter Elvehjem tissue grinder. This was assumed to be similar to the "very loose fitting" pestle described by Auricchio, Mollica and Liguori (3).

The rotational velocity of the drill with no load was set at 690 rpm for all experiments except for 14, 15 and one trial each of 1 and 2, for which it was set at 3800 rpm. In the four cases specified, 5 up/down strokes of the homogenizer were used while in all other experiments 10 up/down strokes were used. No effect of rotational velocity of the drill was observed on the inactivation of tyrosine aminotransferase, and this has not been considered in the interpretation of the data.

The homogenates were centrifuged in a refrigerated centrifuge (model B-20; International Equipment Co.). A fixed angle rotor was used, for which centrifugal force was calculated from the radius measured at the center of the centrifuge tubes.

Except for several experiments, which are specifically noted, the preparation of the liver homogenates was carried out at 0 to 5 C. Further details and variations in the above procedure are provided in the Experimental Design.

D. Incubation of Homogenates

Following centrifugation, the supernatants were rapidly warmed to 37 C by agitating in a hot (approximately 50 C) water bath. The pH was measured at this time and adjusted if necessary. Subsequently, the incubations were set up as quickly as possible in a temperature controlled water bath (37 C).

Two types of incubation vessel were used; a 125 ml Erlenmyer flask in a shaking water bath, or a 100 ml beaker in a stationary water bath fitted over a magnetic stirring unit which allowed stirring of the homogenates. In either mode, the homogenates were agitated throughout the incubation. Each vessel was fitted with inlet and outlet tubes to allow for filtering, sample withdrawal, and atmosphere control.

When required, a continuous stream of N_2 or O_2 was passed over a homogenate during incubation. To prevent evaporation from the homogenate, the gas was first saturated with water at the temperature of the incubation bath. When no control of the atmosphere was required, a small hole was left in the top of the incubation vessel to allow for volume changes. This has been designated as an air atmosphere throughout the thesis. Special attention was usually paid to the control of the gaseous environment because the system used influenced the results. For example, if the incubation vessel was sealed with an O_2 atmosphere, different results were obtained than when O_2 was continuously supplied to the homogenate.

Prior to enzyme analysis, the homogenates were filtered by one of two methods. An "in-line" filter on the inlet end of the homogenate withdrawal line (see Appendix A, Fig. 3 for the function of the homogenate withdrawal line) consisted of a stainless steel Swinny Filter Holder (Millipore) fitted with a filter disc 13 mm in diameter cut from a milk filter (Rapid Flo [guaze faced absorbent cotton]; Johnson and Johnson). The in-line filter was submerged in the homogenate and used only with the Erlenmyer flask as the incubation vessel. Alternatively, an "external" filter consisted of a Swinnex-25 Filter Holder (Millipore) fitted with a filter disc 25 mm in diameter cut from a milk filter. The external filter was submerged in a 37 C water bath and was independent of the homogenate withdrawal line. The homogenate was continuously pumped through the external filter and returned to the incubation vessel with a turnover time of about 20 min for the entire homogenate.

The type of filtering and stirring or shaking system used during the incubation did not have any observable effect on enzyme inactivation, but did have a significant effect on the operation of the equipment. Precipitation of protein in the homogenate during incubation occasionally resulted in plugging of the sample withdrawal line. The lowest incidence of this problem occurred with the beaker system using an external filter.

Incubation periods, during which enzyme analysis was carried out varied from 2.1 to 6.1 h with a mean of 4.5 h. Usually an incubation was shorter if inactivation was rapid. Two short incubations could be performed in one day, one in the morning beginning between 9:00 and 9:30 and one in the afternoon beginning between 1:00 and 1:30. Long incubations began between 9:00 and 9:30 AM, and carried on into the afternoon. No variation in results was attributed to the time of day the incubations were performed.

Each incubation with accompanying measurements of enzyme activity are referred to as a single trial. Each trial required a fresh rat and only one rat was used per trial. An experiment consisted of one trial or more than one trial performed under the same conditions.

E. Enzyme Analyses

Enzyme assays were performed with two techniques based on the tyrosine aminotransferase assay method reported by Diamondstone (17). Individual enzyme assays were carried out using a modification of Diamondstone's method described in Appendix A. Most enzyme assays, though, were done with an automated procedure also described in Appendix A. The automated procedure employed Auto Analyzer (Technicon Instruments Corp.) components and was based on the individual enzyme assay method. With the automated technique it was possible to obtain a continuous record of the enzyme activity in the homogenate.

An evaluation of the validity of the automated method was possible by comparing the results obtained in experiments 1 and 2 (see Table 3). In experiment 2 samples were periodically withdrawn from the homogenate by pipette, diluted and analyzed for tyrosine aminotransferase activity by the individual assay method, while in experiment 1, which differed from experiment 2 only in the assay method, the automated procedure was used.

Because of the addition of pyridoxal 5'-phosphate to the enzyme assay mixture, both methods of tyrosine aminotransferase analysis would measure activity for the apoenzyme as well as the holoenzyme. The recovery of full enzyme activity upon addition of pyridoxal 5'-phosphate to tyrosine aminotransferase apoenzyme has been observed by Hayashi, Granner and Tomkins (37).

F. *Experimental Design*

The experiments performed were of an exploratory nature, such that the results of one experiment or set of experiments influenced the design of subsequent experiments. The details of the experiments performed and the parameters investigated are provided in Table 1.

Initially, the experimental conditions of Auricchio, Mollica and Liguori (3) were repeated as precisely as possible. As a result of these experiments, a number of parameters affecting the inactivation of tyrosine aminotransferase were further investigated. Those parameters investigated were induction time, dose of triamcinolone, pH, atmosphere, buffers, centrifuging out the particulate fraction, preparation temperature of the homogenate and various additions to the homogenate including dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), Mg^{++}, Ca^{++}, cysteine (Sigma Chemical Co.), cystine (Sigma Chemical Co.) and catalysts of cysteine oxidation ($CuSO_4$ and $Na_4P_2O_7$).

Table 1. Experimental design. The preparation of the buffer solutions used is described in the text: a, Krebs-Ringer-phosphate; b, 0.017 M tris: c, 0.030 M tris; d, 0.080 M tris; e, 0.308 M tris-maleate; f, 0.308 M tris-citrate; g, 0.100 M tris-citrate. Solutions containing cysteine, cystine, $CuSO_4$, or $Na_4P_2O_7$ were made up immediately before use. The temperature ranges specified apply to all stages of homogenate preparation. The pH of a homogenate was measured immediately before the beginning of an incubation and at the temperature of the incubation. Any adjustments in the pH were made at this time. The pH of homogenates prepared with Krebs-Ringer-phosphate buffer, however, was not adjusted. The experiments are not presented in chronological order.

		Induction		Homogenization				Incubation		
Expt. no.	No. of trials^1	triamcinolone mg(100 g body wt.)$^{-1}$	induction time h	buffer	additions to buffer	temperature C	centrifugation *g*(min)	atmosphere	initial pH	final pH
1	3	15	10	a		0-5	750(10)	air	7.2^2	7.2^2
2	2	15	10	a		0-5	750(10)	air	7.2^2	7.2^2
3	2	15	16	a		0-5	750(10)	O_2	7.3	7.3
4	2	15	16	a	3 mM DTT^3	0-5	750(10)	O_2	7.1	7.0
5	3	15	10	a	10 mM cysteine	0-5	750(10)	air	7.0^2	6.8^2
6	1	15	11	a	10 mM cysteine	0-5	270(10)	O_2	7.0^2	7.0^2
7	1	15	11	a		0-5	84(5)	N_2		
8	1	15	10	a		0-5	13000(10)	N_2		

Table 1. (cont.)

		Induction		Homogenization				Incubation		
Expt. no.	No. of trials	triamcinolone mg(100 g body wt.)$^{-1}$	induction time h	buffer	additions to buffer	temperature C	centrifugation *g*(min)	atmosphere	initial pH	final pH
9	1	15	11	b		0–5	270(10)	O_2	7.0	7.0
10	1	15	11	b	1 mM DTT	0–5	270(10)	O_2	7.0	
11	1	15	11	b	1 mM EDTA	0–5	270(10)	O_2	7.1	
12	1	15	11	b	1 mM DTT 1 mM EDTA	0–5	270(10)	O_2	7.0	
13	1	15	11	b	1 mM cysteine	0–5	270(10)	O_2	7.0	
14	1	15	11	b	10 mM cysteine	0–5	270(10)	O_2	7.0	6.6
15	1	15	11	b	10 mM cysteine	0–5	270(10)	N_2	7.0	
16	1	15	11	b	10 mM cysteine 0.37 mM $CaCl_2$	0–5	270(10)	O_2	7.0	
17	1	15	11	b	10 mM cysteine 1 mM $MgCl_2$	0–5	270(10)	O_2	7.0	

Table 1. (cont.)

		Induction		Homogenization				Incubation		
Expt. no.	No. of trials	triamcinolone mg(100 g body wt.)$^{-1}$	induction time h	buffer	additions to buffer	temperature C	centrifugation *g*(min)	atmosphere	initial pH	final pH
18	1	15	11	c	10 mM cysteine	0-5	270(10)	O_2	7.0	6.5
19	1	15	11	d	10 mM cysteine	0-5	270(10)	O_2	7.0	6.6
20	1	15	10	e	10 mM cysteine	0-5	270(10)	O_2	7.0	6.8
21	1	15	11	f	10 mM cysteine	0-5	270(10)	O_2	6.5	6.7
22	1	15	11	f	10 mM cysteine	0-5	270(10)	O_2	7.0	7.1
23	1	15	11	f	10 mM cysteine	0-5	270(10)	O_2	7.5	7.5
24	1	15	16	f	10 mM cysteine	0-5	270(10)	O_2	7.5	7.5
25	1	15	11	f	10 mM cysteine	0-5	270(10)	O_2	8.0	8.0
26	1	15	11	f	10 mM cysteine	0-5	270(10)	O_2	8.5	8.5

Table 1. (cont.)

		Induction		Homogenization				Incubation		
Expt. no.	No. of trials	triamcinolone mg(100 g body wt.)$^{-1}$	induction time h	buffer	additions to buffer	temperature C	centrifugation *g*(min)	atmosphere	initial pH	final pH
27	1	15	11	f	10 mM cysteine	0-5	270(10)	O_2	9.0	8.9
28	1	15	11	f	5 mM cystine	0-5	270(10)	O_2	7.5	7.5
29	2	15	16	f	5 mM cystine	0-5	270(10)	O_2	7.5	7.5
30	1	15	20	f	5 mM cystine	0-5	270(10)	O_2	7.5	7.5
31	3	30	16	f	5 mM cystine	0-5	270(10)	O_2	7.5	7.5
32	2	15	16	f	5 mM cystine	0-5	270(10)	O_2	7.5	7.5
33	2	15	16	f	5 mM cystine	0-5	13000(10)	O_2	7.5	7.5
34	1	15	16	f	5 mM cystine 0.02 M $Na_4P_2O_7$	0-5	13000(10)	O_2	7.5	7.5

Table 1. (cont.)

		Induction		Homogenization				Incubation		
Expt. no.	No. of trials	triamcinolone mg(100 g body wt.)$^{-1}$	induction time h	buffer	additions to buffer	temperature C	centrifugation *g*(min)	atmosphere	initial pH	final pH
35	3	15	16	f	5 mM cystine 0.02 M $Na_4P_2O_7$ 0.2 mM $CuSO_4$	0-5	13000(10)	O_2	7.5	7.5
36	2	15	16	f		0-5	270(10)	O_2	7.5	7.6
37	2	15	16	f	10 mM cysteine	0-5	270(10)	N_2	7.5	7.6
38	2	15	16	f	10 mM cysteine	0-5	13000(10)	O_2	7.5	7.4
39	1	15	16	g		0-5	750(10)	O_2	7.5	7.6
40	2	15	16	g		32-37	750(10)	O_2	7.5	7.4
41	1	15	16	g		36-39	270(10)	O_2	7.5	7.6

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[1] One rat was used per trial.

[2] These pH measurements were determined in separate experiments done in duplicate. Mean values are presented.

[3] The amount of solid DTT estimated to make a 3 mM solution was added to the homogenate 50 min after the start of the incubation.

RESULTS

A. General

Triamcinolone is a white powder of low solubility in physiological saline. It was slowly absorbed by the tissues of the rat, and traces were clearly visible in the peritoneal cavity at the time of dissection. In a few cases, however, there was no visible evidence of the hormone and it was assumed that injection may have been into the intestinal lumen. When this occurred, the induced activity of tyrosine aminotransferase was the same as usual, and the experiments were continued without interruption.

The specific activity of tyrosine aminotransferase, after induction by triamcinolone, was found to be 0.43 and 0.57 μkat per g of fresh liver tissue in the two trials of experiment 2. Because dilution factors and flow rates were neither precisely known nor constant from day to day in the automated system, the specific activity was not determined for experiments using the automated procedure. However, any large discrepancies in specific activity between rats would have been immediately apparent. It was not unusual to find a a difference in induced tyrosine aminotransferase activity as high as 100 % between animals from different litters. In order to allow comparisons between experiments, the change in enzyme activity observed during the incubation of homogenates has been expressed as a percent of the original activity.

Tyrosine aminotransferase inactivation was generally patterned after one of the 4 basic curve types represented in Table 2 and Fig. 1. The curve type code defined in Table 2 has been used to describe the shape of inactivation curves obtained. Occasionally, subscripts such as A_B have been used to describe intermediate types.

The results of each inactivation study have been described by the shape of the inactivation curve, the duration of the lag period, the rate of enzyme inactivation during the most rapid phase of inactivation (phase 1) and the average rate of inactivation calculated from the initial and final enzyme activities. Any trial with curve type C or D was terminated if the rate of inactivation decreased to less than 1 % of the original activity per h.

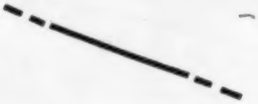
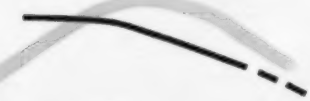
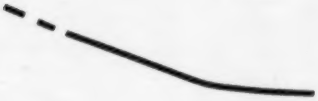
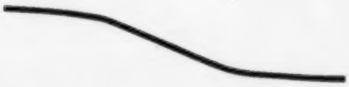
The results of an inactivation experiment entailing individual enzyme assays (expt. 2) were similar to those obtained with the automated method (expt. 1) (Table 3). These results indicated that the automated technique was a viable alternative for the individual tyrosine aminotransferase assay method. Except for experiment 1, all experiments were performed with the automated method, which saved both time and labor compared to the manual method.

B. *Statistical Analysis*

To facilitate statistical analysis several experiments have been grouped together. As discussed above, the assay method did not affect tyrosine aminotransferase inactivation in experiments 1 and 2. These experiments, therefore, have been considered as a single experiment with 5 trials rather than 2 experiments with 3 and 2 trials each.

Experiments 23 and 24 as well as experiments 28, 29 and 30

Table 2. Basic curve types.

Illustration			
lag	phase 1	phase 2	Code[1]
			A
			B
			C
			D

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[1] Subscripts, such as A_B, have been used to denote intermediate curve types. Broken lines have been used to indicate where the terms "lag" and "phase 2" do not apply.

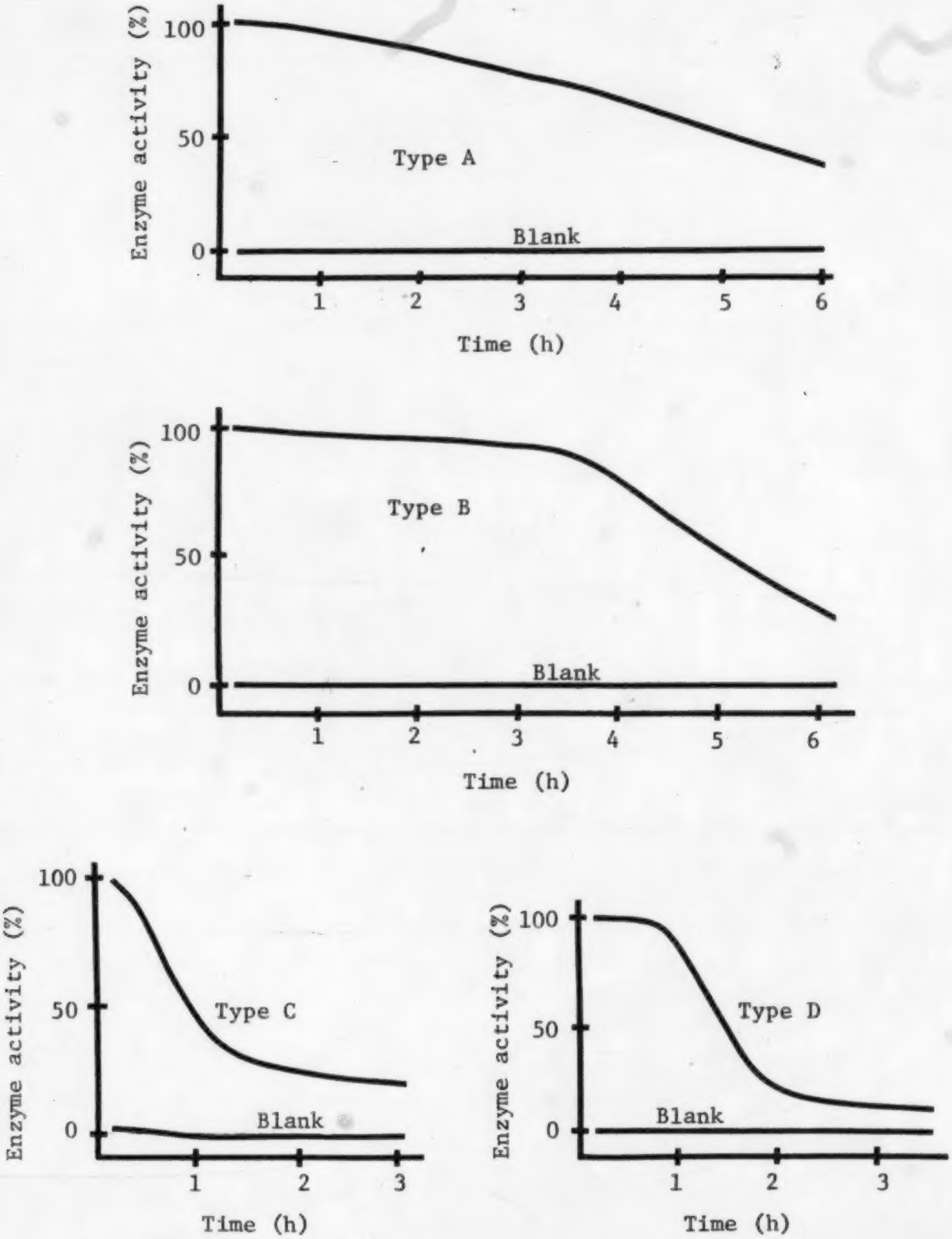

Fig. 1. Examples of curve types obtained during inactivation experiments. Types A, B, C and D are single trials from experiments 40, 38, 31 and 24, respectively.

Table 3. Comparison of automated and manual tyrosine aminotransferase assay methods. Rats received intraperitoneal injections of triamcinolone 10 h before killing. The livers were removed and gently homogenized in 10 volumes of Krebs-Ringer-phosphate buffer (buffer a). The homogenates were centrifuged at 750 × *g* for 10 min and then incubated at 37 C for 3 to 6 h. During this time samples were removed for tyrosine aminotransferase analysis, and changes in enzyme activity were plotted against time. Further experimental details are described in the text and in Table 1. Mean values are given.

			Tyrosine aminotransferase inactivation			
Assay method	Expt. no.	No. of trials	curve type	duration of lag period h	rate of inactivation of phase 1 $\%(h)^{-1}$	average rate of inactivation $\%(h)^{-1}$
manual	1	3	A	0.1	5.0	4.8
automated	2	2	A	0.1	3.9	3.9
Standard deviation $(S_p)^1$				0.2	7.4	2.4

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[1] See text, page 122.

have also been grouped to form two single experiments for statistical analysis. In these experiments the length of the induction time was varied from 11 to 20 h. Since no effect of induction time was observed (Table 4), it has been assumed that induction time had no effect on the inactivation of tyrosine aminotransferase. Further justification for this assumption was obtained from regression analysis, which showed that the variation in the results due to induction time was negligible. Under this assumption a number of comparisons have been made between experiments which differ in induction time between 11 and 20 h in addition to the treatment being compared.

Forty-one experiments were performed with 1 to 3 trials in each experiment. The variation found between trials in all of the experiments was used to calculate pooled standard deviations (S_p) for the duration of the lag period, the rate of inactivation of phase 1 and the average rate of inactivation. The pooled standard deviations are 0.2 h, 7.4 $\%(h)^{-1}$ and 2.4 $\%(h)^{-1}$, respectively.

Plotting the difference from the mean of both the phase 1 and average rates of inactivation for the trials in each experiment against rate of inactivation showed no relationship between inactivation rate and the variation in rate found within experiments. Therefore, the standard deviations for the average and phase 1 rates of inactivation are not a function of the rate of inactivation and may be validly applied to experiments of both slow and rapid inactivation rates.

Multiple comparison of means by Tukey's honestly significant difference (hsd) procedure (110) were performed on 32 comparisons between treatments at the 5 % level of significance. Comparisons were made within average rates of inactivation, rates of inactivation of

Fig. 5. System for collecting $^{14}CO_2$. The two regulating valves (right side of diagram) were set for a flow of 1 $l(min)^{-1}$. Once set, these valves were only adjusted to compensate for fluctuations in flow rate. Starting with the bypass line open and the other stopcocks closed a sample of $^{14}CO_2$ was collected as follows: 8 ml of scintillation fluid was added where indicated; stopcock 3 was opened; then stopcock 1 was opened to the scintillation fluid; when the scintillation fluid had passed into the collection tube, stopcock 1 was closed; at the correct time to begin collecting $^{14}CO_2$, stopcock 2 was turned to the position shown in the diagram; collection continued for 30 min; at the end of 30 min, stopcock 2 was returned to the bypass position; then stopcock 3 was closed and stopcock 1 was opened to N_2; stopcock 4 was opened and the scintillation fluid flowed into the vial while 4 ml of scintillation fluid was added where indicated; then stopcocks 1 and 4 were closed, 3 was opened and after the pressure was reduced in the collection tube (2 or 3 sec) stopcock 1 was opened to the scintillation fluid; the scintillation fluid was washed through the apparatus into the vial; finally, the washing procedure was completed as described in the text. At the end of an experiment, the apparatus was cleaned by rinsing with 95 % ethanol.

Table 4. Effect of induction time on tyrosine aminotransferase inactivation. Before killing, rats recieved intraperitoneal injections of triamcinolone at the times specified in the table. The livers were removed and gently homogenized in 10 volumes of buffer f (0.308 M tris-citrate) containing either 10 mM cysteine (expts. 23 and 24) or 5 mM cystine (expts. 28, 29 and 30). The homogenates were centrifuged at 270 × *g* for 10 min and then incubated at 37 C under an O_2 atmosphere. During incubation tyrosine aminotransferase activity was assayed by the automated method and changes in enzyme activity were plotted against time. Further experimental details are described in the text and in Table 1. Mean values are given where appropriate.

				Tyrosine aminotransferase inactivation		
Induction time h	Expt. no.	No. of trials	curve type	duration of lag period h	rate of inactivation of phase 1 $\%(h)^{-1}$	average rate of inactivation $\%(h)^{-1}$
11	23	1	D	1.1	114	23.6
16	24	1	D	1.0	89.5	21.1
11	28	1	C	0.0	98.0	28.5
16	29	2	C	0.3	89.9	27.2
20	30	1	C	0.3	104	27.1
Standard deviation $(S_p)^1$				0.2	7.4	2.4

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[1] See text, page 122.

phase 1 and the duration of the lag periods. Since the Studentized Range Values used in Tukey's hsd procedure are based on all possible comparisons between treatments, the number of treatments involved in making 32 comparisons was adjusted to 8 as recommended by Cicchette (10).

C. Effect of Dose of Triamcinolone on the Inactivation of Tyrosine Aminotransferase in Rat Liver Homogenates

Valeriote et al. (118) found that intraperitoneal injection of triamcinolone resulted in maximum induction of tyrosine aminotransferase in rat liver at a dose of 10 mg(100 g body wt.)$^{-1}$. These investigators selected a dose of 15 mg(100 g body wt.)$^{-1}$ for routine use. This dose was also used in our experiments. An experiment was performed to test the effect of increasing the dose of triamcinolone on the inactivation of tyrosine aminotransferase. The results (Table 5) show that there was virtually no difference caused in tyrosine aminotransferase inactivation by increasing the triamcinolone treatment from 15 to 30 mg(100 g body wt.)$^{-1}$.

D. Effect of Buffer on the Inactivation of Tyrosine Aminotransferase

Seven different buffers were used for the incubation of rat liver homogenates. Buffer a (Krebs-Ringer-phosphate), buffer b (0.017 M tris) and buffer f (0.308 M tris-citrate) were used extensively while buffer c (0.030 M tris), buffer d (0.080 M tris), buffer e (0.308 M tris-maleate) and buffer g (0.10 M tris-citrate) were used infrequently.

Table 5. Effect of the dosage of triamcinolone on the inactivation of tyrosine aminotransferase in rat liver homogenates. Tyrosine aminotransferase was induced in rat livers by intraperitoneal injections of triamcinolone at the levels specified in the table. After killing, livers were removed and gently homogenized in 10 volumes of buffer f (0.308 M tris-citrate) including 5 mM cystine. The homogenates were centrifuged at 270 × *g* for 10 min and incubated under an O_2 atmosphere. During incubation tyrosine aminotransferase activity was assayed by the automated method and changes in enzyme activity were plotted against time. Further experimental details are described in the text and in Table 1. Mean values are given.

			Tyrosine aminotransferase inactivation			
Triamcinolone mg(100 g body wt.)$^{-1}$	Expt. no.	No. of trials	curve type	duration of lag period h	rate of inactivation of phase 1 %(h)$^{-1}$	average rate of inactivation %(h)$^{-1}$
15	28,29,30	4	C	0.2	95.5	27.5
30	31	3	C_D	0.2	96.6	31.0
Standard deviation (S_p)[1]				0.2	7.4	2.4
Comparison (by expt. no.): 28, 29, 30, vs. 31				-[†]	-	-

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[1] See text, page 122.

[†] In all tables a dash denotes non-significance.

When homogenates were centrifuged at 270 × *g* for 10 min and incubated under an O_2 atmosphere in the presence of 9.1 mM cysteine[1], buffers b, c and d produced rates of inactivation similar to buffer a (Table 6). Buffer e, however, produced a significantly slower rate of enzyme inactivation. Maleate was the only factor in buffer e not common to the other buffers.

Buffers b, c and d did not have sufficient buffering capacity to maintain the pH of the homogenates within a reasonable range (± 0.2 pH units) throughout the incubation period (Table 6). Buffer f was found to be adequate in this respect.

Buffer f completely inhibited enzyme inactivation in the absence of cysteine and presence of O_2, whereas under similar conditions buffer a produced a rate of inactivation of 10.9 % of the original activity per h (Table 7). The inhibition in the presence of buffer f was statistically significant.[2]

E. Effects of O_2, N_2 and Air on the Inactivation of Tyrosine Aminotransferase

The inactivation of tyrosine aminotransferase in rat liver homogenates was affected by the gaseous environment over the homogenate

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[1] Since 10 volumes of buffer were used to homogenize the livers, the components of the buffer solutions were diluted by a factor of 1.1 in the homogenates.

[2] Note from Table 1, however, that these experiments (3 and 36) differed slightly with respect to centrifugation, although this was unlikely to have affected the results.

Table 6. Effect of buffer with cysteine on the inactivation of tyrosine aminotransferase. Tyrosine aminotransferase was induced in rat livers by intraperitoneal injections of triamcinolone. After killing, livers were removed and gently homogenized in 10 volumes of the buffers specified in the table. All of the buffers contained 10 mM cysteine. The homogenates were centrifuged at 270 × *g* for 10 min and then incubated at 37 C under an O_2 atmosphere. During incubation tyrosine aminotransferase activity was assayed by the automated method and changes in enzyme activity were plotted against time.

Further experimental details are described in the text and in Table 1.

			Incubation		Tyrosine aminotransferase inactivation			
Buffer	Expt. no.	No. of trials	initial pH	final pH	curve type	duration of lag period h	rate of inactivation of phase 1 $\%(h)^{-1}$	average rate of inactivation $\%(h)^{-1}$
a (Krebs-Ringer-phosphate)	6	1	7.0	7.0	D	0.8	61.7	22.2
b (0.017 M tris)	14	1	7.0	6.6	D	0.6	59.4	22.2
c (0.030 M tris)	18	1	7.0	6.5	D	1.2	72.0	25.0
d (0.080 M tris)	19	1	7.0	6.6	D	1.0	60.8	27.8
e (0.308 M tris-maleate)	20	1	7.0	6.8	A_B	0.0	5.9	5.9
f (0.308 M tris-citrate)	22	1	7.0	7.1	D	0.8	57.8	20.8
Standard deviation (S_p)[1]						0.2	7.4	2.4
Comparisons (by expt. no.):					6 vs. 14	-	-	-
					6 vs. 18	-	-	-
					6 vs. 19	-	-	-
					6 vs. 20	-	*	*
					6 vs. 22	-	-	-

[1] See text, page 122.

*Significantly different ($P < 0.05$).

Table 7. Effect of buffer without cysteine on the inactivation of tyrosine aminotransferase. Experimental details were the same as described in Table 6 except that cysteine was not included in the buffers, and experiments 3 and 39 were centrifuged at 750 × *g* for 10 min. Mean values are given where appropriate.

			Incubation		Tyrosine aminotransferase inactivation			
Buffer	Expt. no.	No. of trials	initial pH	final pH	curve type	duration of lag period h	rate of inactivation of phase 1 $\%(h)^{-1}$	average rate of inactivation $\%(h)^{-1}$
a (Krebs-Ringer-phosphate)	3	2	7.3	7.3	C	0.0	17.4	10.9
f (0.308 M tris-citrate)	36	2	7.5	7.6	A		0.0	0.0
g (0.100 M tris-citrate)	39	1	7.5	7.6	A	0.0	2.4	2.4
Standard deviation $(S_p)^1$						0.2	7.4	2.4
Comparisons (by expt. no.):					3 vs. 36		-	*
					3 vs. 39	-	-	-

[1] See text, page 122.

*Significantly different ($P < 0.05$).

(Table 8). The mean of experiments 1 and 2 indicates that tyrosine aminotransferase was inactivated at a rate of 4.5 % of the original activity per h when the homogenate was prepared with buffer a and incubated under an air atmosphere. O_2 (expt. 3), rather than air, produced an increase in the rate of inactivation which was not statistically significant.

At pH 7.0 in buffer b with 9.1 mM cysteine a rapid rate of inactivation was obtained when the homogenate was incubated under an O_2 atmosphere (expt. 14). Under N_2, rather than O_2, inactivation was almost completely inhibited (expt. 15). The difference between experiments 14 and 15 was statistically significant with respect to both the average and phase 1 rates of inactivation, but not with respect to the duration of the lag period.[1]

At pH 7.5 in buffer f with 9.1 mM cysteine and under an O_2 atmosphere a phase 1 rate of inactivation of 102 % of the original activity per h was obtained (Table 8, expts. 23 and 24). Under a N_2 atmosphere the rate of inactivation was significantly inhibited and the duration of the lag period was significantly extended. In the presence of cystine, no significant effect due to substitution of N_2 for O_2 was observed in the duration of the lag period or in the average rate of inactivation (Table 8). The rate of inactivation of phase 1, however, was significantly faster in the presence of O_2 than in the presence of N_2.

[1] When the rate of inactivation was low, as in experiment 15, the measurement of the lag period was inaccurate due to the lack of a clear demarcation between the lag period and the beginning of inactivation.

Table 8. Effect of gaseous environment on the inactivation of tyrosine aminotransferase. Tyrosine aminotransferase was induced in rat livers by intraperitoneal injections of triamcinolone. After killing, livers were removed and gently homogenized in 10 volumes of the buffers specified in the table. The homogenates were centrifuged at 750 × *g* (expts. 1, 2, and 3) or 270 × *g* (expts. 14, 15, 23, 24, 28, 29, 30, 32 and 37) for 10 min and then incubated under an O_2, N_2 or air atmosphere. During incubation tyrosine aminotransferase activity was assayed by the automated method and changes in enzyme activity were plotted against time. Further experimental details are described in the text and in Table 1. Mean values are given where appropriate.

				Tyrosine aminotransferase inactivation			
Atmosphere	Buffer + additions	Expt. no.	No. of trials	curve type	duration of lag period h	rate of inactivation of phase 1 $\%(h)^{-1}$	average rate of inactivation $\%(h)^{-1}$
air	a	1,2	5	A	0.1	4.5	4.5
O_2	a	3	2	C	0.0	17.4	10.9
O_2	b + 10 mM cysteine	14	1	D	0.6	59.4	22.2
N_2	b + 10 mM cysteine	15	1	A	0.0[1]	2.9	2.9
O_2	f + 10 mM cysteine	23,24	2	D	1.1	102	22.4
N_2	f + 10 mM cysteine	37	2	B	3.0	33.6	13.8
O_2	f + 5 mM cystine	28,29,30	4	C	0.2	95.5	27.5
N_2	f + 5 mM cystine	32	2	D_C	0.5	66.3	27.8
Standard deviation $(S_p)^1$					0.2	7.4	2.4
Comparisons (by expt. no.):		1,2 vs. 3			-	-	-
		14 vs. 15			-	*	*
		23,24 vs. 37			*	*	*
		28,29,30 vs. 32			-	*	-

[1] In experiment 15 it may be more realistic to consider the entire period of measurement as a lag period. This would be consistent with the results obtained in experiment 37. Note from Table 1 that experiments 15 and 37 differ with respect to pH.

[†] See text, page 122.

*Significantly different ($P < 0.05$).

F. Effects of DTT and EDTA on the Inactivation of Tyrosine Aminotransferase

Both DTT and EDTA appeared to have inhibitory effects on the inactivation of tyrosine aminotransferase when homogenates were incubated without cysteine at pH 7 in buffer b (0.017 M tris) (Table 9). The inhibition in the presence of EDTA was greater than that in the presence of DTT, but neither resulted in a statistically significant reduction in rate of inactivation. EDTA and DTT together had the same effect as EDTA alone.

Cystine and other disulfides are known to cause the inactivation of a number of enzymes (5, 40, 84, 85, 94, 95). Some of these enzymes may be reactivated by incubation with sulfhydryl compounds (5, 40, 94). Since the inactivation of tyrosine aminotransferase may have been due to the presence of disulfides, 3 mM DTT was added 50 min after the start of an incubation under O_2 (expt. 4). However, this treatment had no significant effect on enzyme inactivation (Table 10), and recovery of enzyme activity was not observed.

G. Effect of pH on the Inactivation of Tyrosine Aminotransferase

Fig. 2 illustrates the effect of pH on the rate of tyrosine aminotransferase inactivation in the presence of 9.1 mM cysteine, O_2 and buffer f (expts. 21, 22, 23, 25, 26, 27). With increasing pH, the lag period reached a maximum at pH 7.5 and rapidly decreased to zero at pH 8.5. It appeared to increase again at pH 9, but the unusual shape of the curve indicated that additional factors may be involved at higher pH values. The rate of phase 1 inactivation peaked

Table 9. Effects of DTT and EDTA on inactivation of tyrosine aminotransferase. Tyrosine aminotransferase was induced in rat livers by intraperitoneal injections of triamcinolone. After killing, livers were removed and gently homogenized in 10 volumes of buffer b (0.017 M tris, pH 7.0) including DTT and EDTA as specified in the table. The homogenates were centrifuged at 270 × *g* for 10 min and then incubated at 37 C under an O_2 atmosphere. During incubation tyrosine aminotransferase activity was assayed by the automated method and changes in enzyme activity were plotted against time. Further experimental details are described in the text and in Table 1.

				Tyrosine aminotransferase inactivation		
Additions to buffer	Expt. no.	No. of trials	curve type	duration of lag period h	rate of inactivation of phase 1 $\%(h)^{-1}$	average rate of inactivation $\%(h)^{-1}$
none	9	1	D	0.7	12.4	4.7
1 mM DTT	10	1	A_B		5.3	5.3
1 mM EDTA	11	1	A_B	0.6	1.8	1.6
1 mM DTT + 1 mM EDTA	12	1	A_B	0.3	1.6	1.6
Standard deviation (S_p)[1]				0.2	7.4	2.4
Comparisons (by expt. no.):			9 vs. 10	-	-	-
			9 vs. 11	-	-	-
			9 vs. 12	-	-	-

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[1] See text, page 122.

Table 10. Effect of DTT on recovery of tyrosine aminotransferase activity. Tyrosine aminotransferase was induced in rat livers by intraperitoneal injections of triamcinolone. After killing, livers were removed and gently homogenized in 10 volumes of buffer a (Krebs-Ringer-phosphate). The homogenates were centrifuged at 750 × *g* for 10 min and incubated under an O_2 atmosphere. Fifty min after the start of the incubation, DTT was added to the homogenates of experiment 4 to make an estimated concentration of 3 mM. During incubation tyrosine aminotransferase activity was assayed by the automated method and changes in enzyme activity were plotted against time. Further experimental details are described in the text and in Table 1. Mean values are given.

				Tyrosine aminotransferase inactivation		
Additions to buffer	Expt. no.	No. of trials	curve type	duration of lag period h	rate of inactivation of phase 1 $\%(h)^{-1}$	average rate of inactivation $\%(h)^{-1}$
none	3	2	C	0.0	17.4	10.9
3 mM DTT at 50 min	4	2	A	0.4	20.6	17.5
Standard deviation $(S_p)^1$				0.2	7.4	2.4
Comparison (by expt. no.):		3 vs. 4		-	-	-

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[1] See text, page 122.

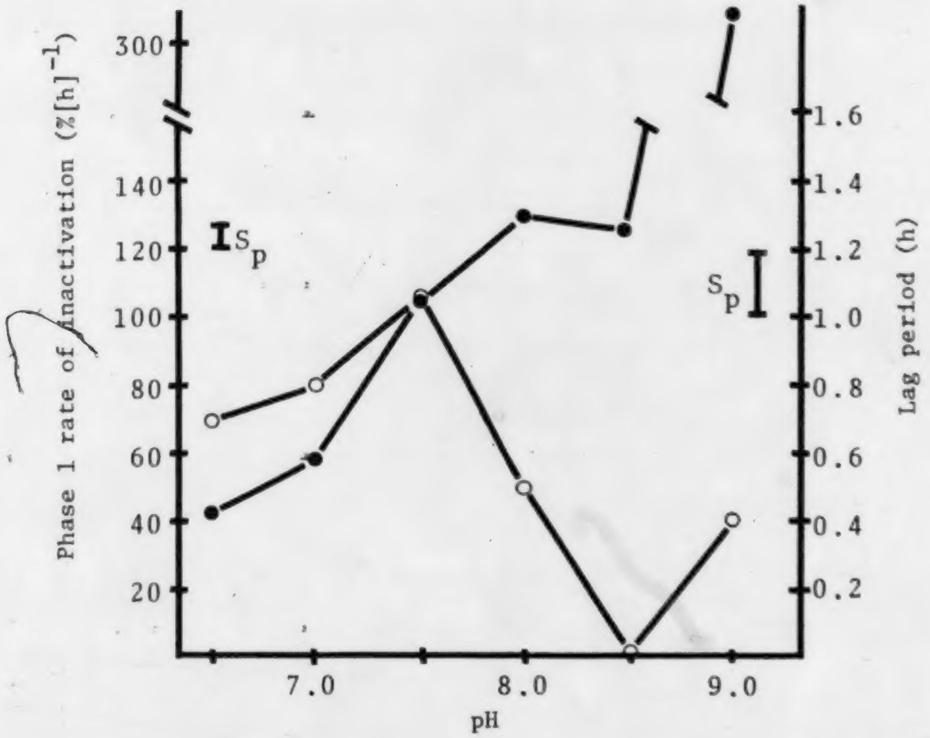

Fig. 2. Effect of pH on the duration of the lag period (○) and on the rate of tyrosine aminotransferase inactivation (●) measured during phase 1.

at pH 8, but an additional large increase was observed between pH 8.5 and 9. Since the lag period appeared to be a significant factor in enzyme inactivation and since the rate of inactivation showed a substantial increase between pH 7 and 7.5, it was decided to perform subsequent experiments at pH 7.5.

H. Effect of Preparation Temperature on the Inactivation of Tyrosine Aminotransferase

Auricchio, Mollica and Liguori (3) concluded that damage to the lysosomal membrane was minimized by gentle homogenization, thus permitting inactivation of tyrosine aminotransferase to occur. Since membranes might be more fragile at 0 to 5 C, the effect of gently homogenizing and centrifuging at 32 to 37 C compared to 0 to 5 C was investigated. As shown in Table 11, the temperature of homogenate preparation had no significant effect on enzyme inactivation.

I. Effects of Cysteine and Cystine on the Inactivation of Tyrosine Aminotransferase

The mean of experiments 1 and 2 indicated that tyrosine aminotransferase was inactivated at a rate of 4.5 % of the original activity per h when the homogenate was prepared with buffer a and incubated under an air atmosphere. The addition of 9.1 mM cysteine (expt. 5) did not increase the rate of inactivation (Table 12). Varying the conditions, however, resulted in more pronounced effects upon the addition of cysteine (Table 12). When homogenates were prepared with buffer b (0.017 M tris, pH 7.0) and incubated under an O_2 atmosphere the addition of 0.9 mM cysteine resulted in a slight increase in enzyme

Table 11. Effect of preparation temperature on the inactivation of tyrosine aminotransferase. Tyrosine aminotransferase was induced in rat livers by intraperitoneal injections of triamcinolone. After killing, livers were removed and gently homogenized in 10 volumes of buffer g. Homogenates were centrifuged at 750 × g for 10 min and incubated at 37 C under an O_2 atmosphere. In experiment 40 all steps in the preparation of the homogenate were carried out at 32 to 37 C, whereas in experiment 39 the homogenate was prepared as usual at 0 to 5 C. During incubation tyrosine aminotransferase activity was assayed by the automated method and the changes in enzyme activity were plotted against time. Further experimental details are provided in the text and in Table 1. Mean values are given where appropriate.

			Tyrosine aminotransferase inactivation			
Preparation temperature C	Expt. no.	No. of trials	curve type	duration of lag period h	rate of inactivation of phase 1 $\%(h)^{-1}$	average rate of inactivation $\%(h)^{-1}$
0-5	39	1	A	0.0	2.4	2.4
32-37	40	2	A	0.0	7.7	6.5
Standard deviation $(S_p)^1$				0.2	7.4	2.4
Comparison (by expt. no.): 39 vs. 40				-	-	-

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[1] See text, page 122.

Table 12. Effects of cysteine and cystine on the inactivation of tyrosine aminotransferase. tyrosine aminotransferase was induced in rat livers by intraperitoneal injections of triamcinolone. After killing, livers were removed and gently homogenized in 10 volumes of buffer including cysteine or cystine as specified in the table. The homogenates were centrifuged at 750 × *g* (expts. 1, 2 and 5) or at 270 × *g* (expts. 9, 13, 14, 36, 23, 24, 28, 29, 30 and 36) for 10 min, and then incubated under an air or O_2 atmosphere. During incubation tyrosine aminotransferase activity was assayed by the automated method and changes in enzyme activity were plotted against time. Further experimental details are described in the text and in Table 1. Mean values are given where appropriate.

		Incubation				Tyrosine aminotransferase inactivation			
Additions to buffer	Buffer	atmosphere	pH	Expt. no.	No. of trials	curve type	duration of lag period h	rate of inactivation of phase 1 $\%(h)^{-1}$	average rate of inactivation $\%(h)^{-1}$
none	a	air	7.2	1,2	5	A	0.1	4.5	4.5
10 mM cysteine	a	air	7.0	5	3	A	0.2	5.5	5.6
none	b	O_2	7.0	9	1	D	0.7	12.4	4.7
1 mM cysteine	b	O_2	7.0	13	1	B	1.2	19.6	9.2
10 mM cysteine	b	O_2	7.0	14	1	D	0.6	59.4	22.2
none	f	O_2	7.5	36	2	A		0.0	0.0
10 mM cysteine	f	O_2	7.5	23,24	2	D	1.1	102	22.4
5 mM cystine	f	O_2	7.5	28,29,30	4	C	0.2	95.5	27.5
Standard deviation $(S_p)^1$							0.2	7.4	2.4
Comparisons (by expt. no.):				1,2 vs. 5			-	-	-
				9 vs. 13			-	-	-
				9 vs. 14			-	*	*
				23,24 vs. 36				*	*
				28,29,30 vs. 36				*	*
				23,24 vs. 28,29,30			*	-	-

[1] See text, page 122.

*Significantly different ($P < 0.05$).

inactivation which was not statistically significant (expt. 13). When 9.1 mM cysteine was added, however, there was a significant increase in both the rate of inactivation of phase 1 and the average rate of inactivation.

Tyrosine aminotransferase inactivation in homogenates prepared with buffer f (0.308 M tris-citrate, pH 7.5) and incubated under an O_2 atmosphere was markedly affected by the addition of cysteine. In the absence of cysteine (expt. 36) no inactivation was observed, whereas in the presence of 9.1 mM cysteine (expts. 23 and 24) a lag period of 1.1 h followed by a rapid rate of inactivation (102 % of the original activity per h) resulted. Both the rate of inactivation of phase 1 and the average rate of inactivation were significantly increased. Since a lag period cannot be measured if there is no inactivation, the statistical significance of the lag period in experiments 23 and 24 cannot be tested by comparison with experiment 36.

The presence of 4.5 mM cystine in homogenates prepared with buffer f (expts. 28, 29 and 30) produced inactivation rates similar to those found with 9.1 mM cysteine. The duration of the lag period obtained with 4.5 mM cystine, however, was significantly shorter than that obtained with 9.1 mM cysteine.

The effects of Mg^{++} and Ca^{++} on tyrosine aminotransferase inactivation in the presence of cysteine were investigated with homogenates prepared with buffer b and incubated under an O_2 atmosphere (Table 13). The addition of Ca^{++} resulted in an increase in the rate of inactivation, while the addition of Mg^{++} resulted in a reduction, but neither change was statistically significant. How-

Table 13. Effects of Mg^{++} and Ca^{++} on the inactivation of tyrosine aminotransferase. Experimental details were the same as described in Table 9 except for the additions to the buffer as specified below.

			Tyrosine aminotransferase inactivation			
Additions to buffer	Expt. no.	No. of trials	curve type	duration of lag period h	rate of inactivation of phase 1 $\%(h)^{-1}$	average rate of inactivation $\%(h)^{-1}$
10 mM cysteine	14	1	D	0.6	59.4	22.2
10 mM cysteine, 0.37 mM $CaCl_2$	16	1	D	1.2	75.9	23.3
10 mM cysteine, 1 mM $MgCl_2$	17	1	D	1.2	31.3	14.2
Standard deviation (S_p)[1]				0.2	7.4	2.4
Comparisons (by expt. no.):			14 vs. 16	-	-	-
			14 vs. 17	-	-	-
			16 vs. 17	-	*	-

[1] See text, page 122.

*Significantly different ($P < 0.05$).

ever, when the results obtained with Ca^{++} were compared with those obtained with Mg^{++} a significant difference was found between the rates of inactivation of phase 1.

J. *Effect of Centrifuging on the Rate of Tyrosine Aminotransferase Inactivation in Rat Liver Homogenates*

Auricchio, Mollica and Liguori (3) found that centrifuging at 13000 × *g* for 10 min inhibited the stimulation of inactivation of tyrosine aminotransferase by cysteine in gently homogenized rat liver homogenates. Centrifuging at 750 × *g* for 10 min had no effect (3). These observations have been further investigated under a variety of conditions (Table 14).

Centrifuging at 13000 × *g* for 10 min (expt. 8) did not reduce the already slow rate of inactivation obtained under a N_2 atmosphere (expt. 7).

The difference between centrifuging at 750 × *g* and 270 × *g* was investigated in experiments 40 and 41. With homogenates prepared at 32 to 39 C without any additions and incubated under O_2 the rate of inactivation of tyrosine aminotransferase was found to be slower when centrifuged at 270 × *g* for 10 min than when centrifuged at 750 × *g* for 10 min. The difference was not significant.

Centrifuging at 13000 × *g* (Expt. 38) significantly reduced the rate of enzyme inactivation in the presence of cysteine and O_2 and significantly increased the duration of the lag period when compared to identical homogenates centrifuged at 270 × *g* (expts. 23 and 24).

The rate of tyrosine aminotransferase inactivation in the

Table 14. Effect of centrifuging on the inactivation of tyrosine aminotransferase in rat liver homogenates. Tyrosine aminotransferase was induced in rat livers by intraperitoneal injections of triamcinolone. After killing, livers were removed and gently homogenized in 10 volumes of buffer including additional factors as specified in the table. The homogenates were centrifuged at the forces and times specified and then incubated under an O_2 or N_2 atmosphere. During incubation tyrosine aminotransferase activity was assayed by the automated method and changes in enzyme activity were plotted against time. Further experimental details are described in the text and in Table 1. Mean values are given where appropriate.

							Tyrosine aminotransferase inactivation		
Centrifugation *g*(min)	Buffer	Additions to buffer	Atmosphere	Expt. no.	No. of trials	curve type	duration of lag period h	rate of inactivation of phase 1 $\%(h)^{-1}$	average rate of inactivation $\%(h)^{-1}$
84(5)	a	none	N_2	7	1	A_B	0.5	2.2	2.2
13000(10)	a	none	N_2	8	1	B	0.6	3.9	2.5
270(10)	f	10 mM cysteine	O_2	23,24	2	D	1.1	102	22.4
13000(10)	f	10 mM cysteine	O_2	38	2	B	3.4	31.7	11.3
270(10)	f	5 mM cystine	O_2	28,29,30	4	C	0.2	95.5	27.5
13000(10)	f	5 mM cystine	O_2	33	2	D	1.0	81.0	27.6
750(10)	g	none	O_2	40	2	A	0.0	7.7	6.5
270(10)	g	none	O_2	41	1	A	0.0	1.1	1.1
Standard deviation (S_p)[1]							0.2	7.4	2.4
Comparisons (by expt. no.):					7 vs. 8		–	–	–
					23,24 vs. 38		*	*	*
					28,29,30 vs. 33		*	–	–
					40 vs. 41		–	–	–

[1] See text, page 122.

*Significantly different ($P < 0.05$).

presence of cystine was only slightly affected by centrifuging at 13000 × *g* for 10 min, but the duration of the lag period was significantly increased from 0.2 to 1.0 h (expts. 28, 29, 30 and 33).

The increase in the lag period observed after centrifuging homogenates containing cystine was further investigated (Table 15). Since there may have been some cysteine unpurity in the cystine used to prepare the homogenates and since some reduction of cystine to cysteine would probably occur during the relatively anaerobic preparation of the homogenates, reagents were added which catalyze the oxidation of cysteine to cystine. $Na_4P_2O_7$ and $CuSO_4$ together are known to be effective catalysts (54). The addition of $Na_4P_2O_7$ alone had no effect, but the addition of both $Na_4P_2O_7$ and $CuSO_4$ resulted in a significant reduction in the lag period and a significant increase in the rate of inactivation.

Table 15. Effects of Cu^{++} and $P_2O_7^{4-}$ on the inactivation of tyrosine aminotransferase in 13000 × *g* supernatants of rat liver homogenates. Tyrosine aminotransferase was induced in rat livers by intraperitoneal injections of triamcinolone. After killing, livers were removed and gently homogenized in 10 volumes of buffer f including 5 mM cystine. The homogenates were centrifuged at 13000 × *g* for 10 min and incubated under an O_2 atmosphere. During incubation tyrosine aminotransferase activity was assayed by the automated method and changes in enzyme activity were plotted against time. Further experimental details are described in the text and in Table 1. Mean values are given where appropriate.

			Tyrosine aminotransferase inactivation			
Additions to buffer	Expt. no.	No. of trials	curve type	duration of lag period h	rate of inactivation of phase 1 $\%(h)^{-1}$	average rate of inactivation $\%(h)^{-1}$
5 mM cystine	33	2	D	1.0	81.0	27.6
5 mM cystine, 0.02 M $Na_4P_2O_7$	34	1	D	0.7	69.6	30.4
5 mM cystine, 0.02 M $Na_4P_2O_7$, 0.2 mM $CuSO_4$	35	3	C	0.0	116	51.9
Standard deviation $(S_p)^1$				0.2	7.4	2.4
Comparisons (by expt. no.):			33 vs. 34	–	–	–
			33 vs. 35	*	*	*

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[1] See text, page 122.

*Significantly different ($P < 0.05$).

DISCUSSION

Hershko and Tomkins (38) have recently suggested that tyrosine aminotransferase may be a suitable model for the study of protein degradation. A rapid rate of turnover, marked response to various stimuli and the large amount of information available about the enzyme all contribute to its suitability as a model. Because of the stability of tyrosine aminotransferase after the disruption of the cell, though, there are few studies of its inactivation or degradation in cell-free systems.

A. Results Obtained Under Conditions Described by Previous Workers

Recently, Auricchio, Mollica and Liguori (3) reported the inactivation of tyrosine aminotransferase in crude liver homogenates. Inactivation occurred with a half-life of 8 h if the livers were "gently" homogenized with a "very loose fitting" Teflon pestle in a Potter Elvehjem tissue grinder. Drastic homogenization or removal of the particulate fraction from a gently homogenized liver preparation resulted in no inactivation (3). Krebs-Ringer-phosphate buffer was used as the homogenizing medium and the atmosphere over the homogenates, although not specified, was assumed to be air.

A number of experiments were conducted in an attempt to reproduce and further study some of the results of Auricchio, Mollica and Liguori (3). In the experiments presented here, homogenization was sufficiently gentle as to not disrupt erythrocytes trapped in the liver tissue, as was indicated by the lack of red colour in the centrifuged homogenates and the red band consistently observed in the centrifuged pellets. Homogenates prepared with a tight fitting pestle were reddish after centrifuging and showed no red band in the pellet. After gentle homogenization, a rate of inactivation which was 73 % of that reported by Auricchio, Mollica and Liguori (3) was observed (Table 12, expts. 1 and 2).

Auricchio, Mollica and Liguori (3) found that the addition of cysteine resulted in a 4 fold increase in the rate of inactivation in a gently homogenized preparation. Using similar preparative procedures, it was found (Table 12, expt. 5) that the addition of cysteine had no effect on the inactivation of tyrosine aminotransferase. Although the homogenates were probably incubated under an air atmosphere in both cases, the discrepancy in results may reflect the availability of O_2 to the homogenates. The present experiments have shown that O_2 was required for the cysteine-mediated stimulation of tyrosine aminotransferase inactivation (Table 8). In experiments 1 and 2 there was virtually no air exchange over the homogenates, whereas in the experiments of Auricchio, Mollica and Liguori (3) there may have been free exchange of air, thus maintaining a supply of O_2, and permitting inactivation to occur.

B. Effect of Buffer on the Inactivation of Tyrosine Aminotransferase

Krebs-Ringer-phosphate buffer (buffer a) was used in experiments in which the experimental conditions of Auricchio, Mollica and Liguori (3) were carefully repeated. Hayashi, Granner and Tomkins (37), though, have reported that phosphate competitively inhibited the binding of pyridoxal 5'-phosphate and pyridoxamine to tyrosine aminotransferase. Since the binding of cofactors is known to affect the stability of enzymes, a variety of buffers without phosphate were substituted for Krebs-Ringer-phosphate in subsequent experiments. Several of the buffers resulted in an unacceptable degree of pH drift during incubation. Buffer f (0.308 M tris-citrate), though, was found to adequately control the pH.

During the course of trying different buffers it was found that the maleate in buffer e (0.308 M tris-maleate) significantly inhibited the inactivation of tyrosine aminotransferase in the presence of cysteine. Although the effect of maleate was not further investigated, a possible explanation for the observed inhibition may be its tendency to chelate with Cu^{++} (88) and possibly other ions. Since the inactivation of tyrosine aminotransferase may have been inhibited (although not statistically significant) by the presence of EDTA (table 9), and since the addition of trace amounts of Cu^{++} in the presence of cystine significantly increased the rate of inactivation (Table 15), the chelation of Cu^{++} or other metal ions in the homogenate may explain the inhibitory effect of maleate.[1]

C. The Relationship Between Cystine, Cysteine and O_2

It was found that the addition of O_2 produced a significant increase in the rate of inactivation of tyrosine aminotransferase in the presence of cysteine (Table 8). In the absence of cysteine, O_2 caused a non-significant increase in the rate of inactivation. The results showed, therefore, that both O_2 and cysteine were required for the rapid inactivation of tyrosine aminotransferase.

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[1] It also recognized that citrate is frequently used as a chelating agent for divalent metal ions. However, considerably faster inactivation rates were obtained in the presence of citrate than in the presence of maleate (Table 6, expts. 20 and 22).

Since the oxidation of cysteine is catalyzed by cytochrome c and cytochrome oxidase (54) and also by rat liver mitochondria (119), it is likely that cysteine was oxidized in rat liver homogenates. If so, then cystine, rather than cysteine, may have been the factor evoking tyrosine aminotransferase inactivation.

When cystine was added to homogenates incubated under O_2, tyrosine aminotransferase was rapidly inactivated. When compared to the effect of cysteine, the lag period was significantly reduced while the rate of inactivation remained unchanged (Table 12). When O_2 was replaced by N_2 over the homogenates containing cysteine the rate of inactivation was significantly reduced and the lag period was significantly extended (Table 8). Over homogenates containing cystine, however, N_2 significantly reduced the rate of phase 1 inactivation but did not affect the lag period or the average rate of inactivation (Table 8). Except for the reduction in phase 1 inactivation when N_2 was substituted for O_2 in the presence of cystine (an explanation for this exception is suggested below), these results indicated that cystine was able to replace cysteine and O_2 in the inactivation of tyrosine aminotransferase. Thus, the observed requirement for O_2 in the presence of cysteine may be explained by the oxidation of cysteine prior to enzyme inactivation.

In contrast to this interpretation, Auricchio, Mollica and Liguori (3) concluded that the stimulatory effect of cysteine on the inactivation of tyrosine aminotransferase in rat liver homogenates was due to the activation of cathepsins B and B_1, which are sensitive to the presence of sulfhydryl compounds. Auricchio, Mollica and Liguori (3) did not consider the possibility of cysteine oxidation in their investigations.

As indicated above, one of the present observations also appeared to contradict the interpretation proposed here. It was observed that O_2 had a stimulatory effect on inactivation in the presence of cystine (Table 8). If some cysteine was present and if cysteine inhibited inactivation of tyrosine aminotransferase by cystine (see sections D and F of Discussion), then the stimulatory effect of O_2 may be explained by the oxidation of the cysteine. There may have been some cysteine impurity in the cystine, or some of the added cystine may have been reduced by an exchange reaction with GSH from the liver tissue. This observation, then, may not be inconsistent with anaerobic inactivation of tyrosine aminotransferase by cystine.

The unexpected initiation of inactivation after 3 h of incubation with cysteine under N_2 (Table 8) may have been due, in part, to reactions of cysteine other than oxidation. It seems unlikely that significant oxidation of cysteine could have occurred under a N_2 atmosphere (the N_2 was 99.993 % pure according to specifications) during 3 h of incubation. However, trace amounts of Cu^{++} and various other metal ions catalyze the oxidation of cysteine (54) and may have caused some oxidation in the buffer solution before homogenization. In addition, some oxidation of cysteine may have occurred during homogenate preparation before anaerobic conditions were imposed. The cystine produced would likely have been ineffective in causing tyrosine aminotransferase inactivation in the presence of excess cysteine (see sections D and F of Discussion). During incubation under N_2, though, the excess cysteine may have been catabolized, as was indicated by a strong odour of H_2S over the homogenates.[1] Since the odour was no longer detectable at the end of the incubation the complete catabolism of free cysteine may have occurred. If the concentration of cysteine was sufficiently reduced after 3 h of incubation the cystine remaining in the homogenate may then have caused the inactivation of tyrosine aminotransferase.

Several attempts were made at establishing a method for the estimation of cysteine and cystine in homogenates, but reliable results could not be obtained. Therefore, the changes in cysteine and cystine during incubation have not been determined.

D. *The Inactivation of Enzymes by Mixed Disulfide Formation*

Several enzymes are known to be inactivated by cystine and other disulfides. Inorganic pyrophosphatase (pyrophosphate phosphohydrolase, EC 3.6.1.1), glyceraldehyde-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate:NAD^+ oxidoreductase [phosphorylating], EC 1.2.1.12) and glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:$NADP^+$ 1-oxidoreductase, EC 1.1.1.49) were inactivated by oxidized glutathione (GSSG) in hemolysates (94). Purified hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) and glyceraldehyde-phosphate dehydrogenase were also inactivated by GSSG (95). In other studies (84), however, glyceraldehyde-phosphate dehydrogenase was moderately inactivated by cystamine monosulfoxide, but not by cystamine, GSSG and cystine. The essential sulfhydryl group of papain (EC 3.4.22.2) reacted with cystamine forming an inactive complex (85). Fumarate hydratase (L-malate hydro-lyase, EC 4.2.1.2) reacted with a number of disulfides, including cystine, with a loss of enzyme activity which was proportional to the loss of reactive thiol groups (40). An enzymatically inactive form of purified lysozyme (mucopeptide N-acetylmuramoyl-hydrolase, EC 3.2.1.17) has also been prepared by reaction with cystine (5).

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[1] The odour of H_2S evolved from homogenates containing cysteine when incubated under N_2. It was particularly noticable at pH 7.5. H_2S was likely a product of the reaction catalyzed by cystathionine δ-lyase (L-cystathionine cysteine-lyase, EC 4.4.1.1). This enzyme is found in rat liver (107), and catalyzes the release of H_2S from cysteine.

The inactivation of papain (85), fumarate hydratase (40) and lysozyme (5) was attributed to mixed disulfide formation between the low molecular weight disulfide and the enzyme molecule. Although not confirmed, the other enzymes were apparently inactivated by the same mechanism. The enzyme-disulfide derivatives were generally considered to the the product of the following type of reaction (5).

$$\text{Protein-S}^- + \text{R-S-S-R} \rightarrow \text{Protein-S-S-R} + \text{R-S}^-$$

The inactivation of tyrosine aminotransferase resulting from the presence of cystine in the present experiments may have been due to the same type of reaction; the formation of a mixed disulfide with the enzyme molecule. Evidence that reactive sulfhydryl groups are in or near the reactive site of tyrosine aminotransferase was obtained by Kenney and co-workers (42,55). Iodoacetate, *o*-iodosobenzoate and *p*-chloromercuriphenylsulfonate, which react with protein sulfhydryl groups, were found to cause complete inhibition of tyrosine aminotransferase (55). Also, pyridoxal 5'-phosphate could protect against reaction with the sulfhydryl groups essential for enzyme activity. Although it remains to be proven that tyrosine aminotransferase may be inactivated by mixed disulfide formation between cystine and the sulfhydryl groups at the active site, the results of the present experiments indicate that this may have occurred in the homogenates studied.

Following inactivation by mixed disulfide formation, a few enzymes have been reactivated by incubation with sulfhydryl compounds. Inorganic pyrophosphatase was reactivated by the addition of cysteine (94), but neither glyceraldehyde-phosphate dehydrogenase or glucose-6-phosphate dehydrogenase responded to the same treatment (94). Fully inactivated fumarate hydratase was completely reactivated by prolonged dialysis against mercaptoethanol (40). Lysozyme activity was recovered from the disulfide form by reaction with cysteine, mercaptoethanol or mercaptoethylamine (5). In total, three out of five enzymes were reactivated after incubation with sulfhydryl compounds.

The reversal of mixed disulfide formation by cysteine and other sulfhydryl compounds indicates that mixed disulfide formation would be inhibited in the presence of cysteine. If this is the case then the lag periods observed under both O_2 and N_2 atmospheres may have been due to the presence of cysteine. Inactivation of tyrosine aminotransferase by cystine may not have been possible until the cysteine was either oxidized or catabolized by other reactions.

An attempt to reactivate tyrosine aminotransferase was made in experiment 4 (Table 10). Fifty min after incubation began, the homogenate was made 3 mM in DTT. The addition of DTT, however, neither resulted in recovery of enzyme activity nor inhibition of subsequent inactivation. Although, unsuccessful, this result does not mean that tyrosine aminotransferase could not be reactivated under different conditions.

E. *Mixed Disulfides In Vivo*

Tissue proteins have been found in the form of mixed disulfides with low molecular weight sulfhydryl compounds (35, 70). Recently, Harrap et al. (35) reported that mixed disulfide linkages between protein and GSH or cysteine were found in a variety of rat tissues. Thirty to fifty percent of the total low molecular weight sulfhydryl compounds were found as mixed disulfides and could be released by reduction with sodium borohydride. Similarly, Modig (70) found that the reduction of disulfide bonds of Ehrlich ascites tumour cell proteins released sufficient GSH and other unidentified low molecular weight thiols to account for 30 % of the protein disulfide bonds. Approximately 25 % of the released thiols was GSH (70).

The mechanism of formation of intracellular mixed disulfides has not been investigated, but may be a result of an exchange reaction with low molecular weight disulfide compounds as described in section D. It has not been determined if the formation of mixed disulfides in vivo is associated with intracellular protein catabolism. However, since mixed disulfide formation may have been the mechanism of inactivation of tyrosine aminotransferase in the present experiments the compatability of disulfide inactivation with in vivo conditions has been considered in the following paragraphs.

Inactivation of tyrosine aminotransferase in vivo by an exchange reaction with low molecular weight disulfides may be limited by the availability of disulfide compounds. The in vivo concentration of low molecular weight disulfides has been estimated to be 0.2 μmoles$(g)^{-1}$ in spleen and 0.03 μmoles$(g)^{-1}$ in liver tissue (108). Converting directly to molar concentrations yields approximately 0.2 mM disulfide in spleen and 0.03 mM disulfide in liver. The concentration of free low molecular weight sulfhydryl compounds was found to be considerably higher; 3.4, 7.6 and 0.6 μmoles$(g)^{-1}$ for spleen, liver and blood, respectively (108). The predominant free sulfhydryl compound was GSH (108). Since GSH is known to be readily oxidized in rat liver homogenates (9, 61), GSSG should have been formed in the present experiments. Thus, during incubation in the presence of O_2 the endogenous sulfhydryl and disulfide compounds could have yielded a total concentration of low molecular weight disulfide compounds of about 0.35 mM in the homogenates, allowing for the 11 fold dilution of tissue during homogenization.

In experiment 9, without any addition of cysteine or cystine, an average rate of inactivation of 4.7 % of the original activity per h was observed. When cysteine was added to 0.91 mM (expt. 13), the total disulfide concentration inclusive of endogenous level in the homogenate would have been about 0.8 mM if all of the sulfhydryls were oxidized. This would be approximately twice that expected in experiment 9. Under these conditions an average rate of inactivation of 9.2 % of the original activity per h was obtained. In experiment 6 the addition of 9.1 mM cysteine could produce a total disulfide concentration in the homogenate of about 4.9 mM if there was complete oxidation of cysteine. In this experiment an average rate of inactivation of 22.2 % of the original activity per h was observed. The trend indicated by these results suggests that the concentration of low molecular weight disulfide compounds found in liver tissue in vivo would be too low to cause a significant rate of tyrosine aminotransferase inactivation.

Within intact liver cells, though, additional factors could influence tyrosine aminotransferase inactivation by disulfide compounds. Brostrom and Jeffay (6) concluded that structurally intact tissue was required for protein degradation to take place. Similarly, Schimke, Sweeney and Berlin (100) found that the inactivation of tyrosine aminotransferase was inhibited if cellular structure was disrupted. Within intact liver cells disulfide compounds may be concentrated in certain areas or organelles. If this were the case, then tyrosine aminotransferase inactivation could occur in areas of high disulfide concentration.

An alternative possibility is suggested by the observation that tyrosine aminotransferase exists predominantly as the holoenzyme in rat liver homogenates (63). Since tyrosine aminotransferase apoenzyme is more susceptible to inactivation by heat (37) and by proteolysis (4) than the holoenzyme, the apoenzyme may also be more susceptible to inactivation by mixed disulfide formation. Thus, low concentrations of disulfide may not inactivate tyrosine aminotransferase in homogenates where the enzyme exists as the holoenzyme, but may cause inactivation in vivo if the apoenzyme is formed under conditions of tyrosine aminotransferase degradation.

F. *The function of the Particulate Fraction*

Auricchio, Mollica and Liguori (3) found that cellular particles sedimenting at 13000 × *g* for 10 min were required for the inactivation of tyrosine aminotransferase. The present results confirm those of Auricchio, Mollica and Liguori (3) for homogenates incubated with cysteine. Under these conditions sedimentation of the particulate fraction by centrifuging at 13000 × *g* for 10 min resulted in a significant reduction in the average and phase 1 rates of inactivation and an increase in the duration of the lag period from 1.1 to 3.4 h (Table 14).

In the presence of cystine, however, centrifugation had much less effect on the inactivation of tyrosine aminotransferase. The rate of inactivation was essentially unchanged while the lag period was increased from 0.2 to 1.0 h (Table 14). Therefore, the particulate fraction affected the cysteine-mediated inactivation of tyrosine aminotransferase much more than the cystine-mediated inactivation.

Results with whole homogenates discussed in section C indicated that cysteine was oxidized to cystine prior to tyrosine aminotransferase inactivation. Mitochondria, which should be sedimented by centrifuging at 13000 × *g* for 10 min (106), are known to catalyze the oxidation of cysteine (119). Centrifugation, therefore, would be expected to impair the oxidation of cysteine in the homogenates, and consequently, the inactivation of tyrosine aminotransferase.

In contrast, Auricchio, Mollica and Liguori (3) concluded that the inactivation of tyrosine aminotransferase was stimulated by cysteine because of its effect on the activity of cathepsins B and B_1, and that centrifugation inhibited inactivation due to the concomitant removal of cathepsins B and B_1 with the lysosomal fraction.[1]

The effect of the particulate fraction on the duration of the lag period in centrifuged homogenates containing cystine (Table 14) was further investigated (Table 15). The addition of $Na_4P_2O_7$ and $CuSO_4$, which are known to catalyze the oxidation of cysteine (54), resulted in the elimination of the lag period and a significant increase in the rate of inactivation. This result indicated that cysteine or other low molecular weight sulfhydryl compounds possibly of endogenous origin were causing the inhibition of cystine-mediated inactivation of tyrosine aminotransferase.[1] Because the oxidation of these sulfhydryl compounds would be inhibited by the removal of the particulate fraction the increase in lag period as a result of centrifuging homogenates containing cystine is not inconsistent with the proposed role of the particulate fraction in the cysteine-mediated inactivation of tyrosine aminotransferase.

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[1] In addition to centrifugation at 13000 × *g*, Auricchio, Mollica and Liguori (3) also observed that rigorous homogenization inhibited tyrosine aminotransferase inactivation.. Further work on the rigor of homogenization is required but was not investigated in the present experiments.

G. *Alternative Interpretations*

Because only crude rat liver homogenates were studied it is possible that several mechanisms of inactivation of tyrosine aminotransferase were being observed in the present experiments. Inactivation in the absence of cysteine or cystine may have been due to a mechanism distinct from inactivation in the presence of cysteine or cystine. In addition, pyridoxal 5'-phosphate may have played a role in the inactivation of tyrosine aminotransferase under some conditions.

Except in buffer f, a slow rate of tyrosine aminotransferase inactivation occurred without added cysteine or cystine. Although a separate mechanism may have been observed, several arguments suggest that the mechanism of inactivation was the same as in the presence of cysteine or cystine. Preliminary experiments with chicken liver homogenates, although not reported in this thesis, demonstrated that the characteristics of cysteine-mediated tyrosine aminotransferase inactivation were similar to those found with the rat liver system. In addition, when GSH was substituted for cysteine in the chicken liver homogenates, the results were almost identical to those obtained with cysteine. The concentration of endogenous GSH in the rat liver homogenates would likely have been in the range of 0.5 to 1.0 mM. Since GSH is rapidly oxidized in rat liver homogenates (61), oxidation of endogenous GSH and subsequent mixed disulfide formation with tyrosine aminotransferase could account for the observed inactivation in the absence of cysteine or cystine. Moreover, inactivation both in the absence and presence of cysteine was stimulated by O_2, indicating that the same mechanism may have been operative in both cases.

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[1] Some sulfhydryl-disulfide exchange would be expected to take place between added cystine and endogenous GSH producing cysteine, GSSG and mixed disulfides according to reactions described by Cecil (8).

The results obtained with buffer f were an exception since no inactivation was observed in the absence of cysteine or cystine. The fact that buffer f caused complete inhibition in the absence of those amino acids but permitted inactivation after their addition may suggest that inactivation proceeded by more than one mechanism. However, the added cystine or cysteine may simply have overcome the inhibitory effect of buffer f.

The effect of cysteine on the inactivation of tyrosine aminotransferase may be related to its ability to form a relatively stable complex with pyridoxal 5'-phosphate (7). The complex, which contains a thiazolidine ring (7, 39), forms over a wide range of pH values including pH 7 (7). Since pyridoxal 5'-phosphate appears to have a stabilizing effect on tyrosine aminotransferase (4, 37, 42), the formation of a complex between pyridoxal 5'-phosphate and cysteine might be expected to influence the stability of the enzyme in rat liver homogenates.

Pestaña, Sandoval and Sols (82) have reported the inhibition of tyrosine aminotransferase by homocysteine when this compound was preincubated with the enzyme before the addition of substrate to the assay mixture. Similar inhibition of serine dehydratase was shown to be competitive with pyridoxal 5'-phosphate and was apparently due to complex formation between homocysteine and pyridoxal 5'-phosphate (82). The formation of the complex was investigated and it was found that complex formation could be reversed by gassing with O_2, presumably due to the oxidation of homocysteine to homocystine (82). Thus, the cysteine-pyridoxal 5'-phosphate complex that might have been formed in the present homogenates may also be dissociated by the oxidation of cysteine. Prior to oxidation of the complex, however, tyrosine aminotransferase may have been more susceptible to inactivation since it would likely have been in the apoenzyme form.

Holten, Wicks and Kenney (42) found that rat liver extracts (105,000 × *g* supernatant fraction), which had been dialyzed against 0.05 M phosphate buffer, pH 7.3, rapidly lost tyrosine aminotransferase activity when incubated with 1 mM each of cysteine, histidine and threonine at pH 8.3. Cysteine alone did not cause enzyme inactivation. The addition of 0.06 mM pyridoxal 5'-phosphate delayed the amino acid-initiated enzyme inactivation for 30 min, after which inactivation proceeded at the same rate as in the absence of added coenzyme. In the absence of the addition of both amino acids and pyridoxal 5'-phosphate, tyrosine aminotransferase was relatively stable in the liver extracts. It was found, however, that purified apoenzyme rapidly lost activity at pH 8.7 even in the absence of added amino acids. Furthermore, pyridoxal 5'-phosphate protected against inactivation of tyrosine aminotransferase apoenzyme both in the presence and absence of amino acids, and the addition of amino acids in the absence of pyridoxal 5'-phosphate caused no further increase in the rate of apoenzyme inactivation. From these results it was concluded (42) that the effect of the amino acids was to complex pyridoxal 5'-phosphate causing the apoenzyme to become inactivated because of its inherent instability.

The characteristics of tyrosine aminotransferase inactivation observed by Holten, Wicks and Kenney (42) were different from those observed in the present experiments. It is reported here that both O_2 and the rat liver particulate fraction were required for cysteine-mediated inactivation to proceed, whereas neither were required in the investigations of Holten, Wicks and Kenney (42). However, their experiments were performed at pH 8.3 to 8.7 and the present experiments were performed at pH 7.0 to 7.5; the difference in pH may have had a significant effect on results. In the experiments reported here the rate of inactivation of tyrosine aminotransferase was greatly increased above pH 8.5 (Fig. 2) Although Holten, Wicks and Kenney (42) found that purified tyrosine aminotransferase apoenzyme was unstable at pH 8.7, it was stable at pH 7.5. Thus, the inactivation of tyrosine aminotransferase observed by Holten, Wicks and Kenney (42) may have been similar to that observed in the present experiments above pH 8.5, whereas the inactivation at pH 7.5 reported here probably proceeded by a mechanism other than the inherent instability of the apoenzyme.

H. A Recent Report and Its Implications

After the experiments presented in this thesis were completed, Reynolds and Thompson (92) described the inactivation of tyrosine aminotransferase by cysteine in rat liver homogenates. Following tyrosine aminotransferase induction by the intraperitoneal injection of dexamethasone phosphate, rat livers were homogenized in a loose-fitting Dounce glass homogenizer with 4 volumes of Krebs-Ringer-phosphate buffer at pH 7.0. Homogenates were incubated under an air atmosphere and samples were removed for enzyme analysis at hourly intervals. In the presence of 8 mM cysteine there was a lag period of 1 h followed by an exponential loss of tyrosine aminotransferase activity with a half-life of 0.72 h. It was also observed that centrifuging of homogenates at 105,000 × *g* for 30 min inhibited the inactivation of tyrosine aminotransferase. These results are similar to those reported in this thesis when homogenates contained cysteine and were incubated under O_2. Although the effect of O_2 was not investigated by Reynolds and Thompson (92), there may have been sufficient air exchange over the surface of the homogenates to supply O_2, if it was required for tyrosine aminotransferase inactivation in their experiments.

Other sulfur containing compounds tested by Reynolds and Thompson (92), including cystine, GSSG, GSH and DTT, were not effective in causing the inactivation of tyrosine aminotransferase. The stability of the enzyme in the presence of cystine does not agree with the results presented here. Nor does the stability of the enzyme in the presence of GSH agree with the present observation that chicken liver tyrosine aminotransferase was inactivated in the presence of GSH (section H of Discussion). The discrepancy in results may have been caused by differences in conditions under which the effects of cystine and GSH were investigated. Reynolds and Thompson (92) prepared homogenates in 4 volumes of Krebs-Ringer-phosphate buffer at pH 7.0 and incubated under an air atmosphere. In the present experiments, homogenates were prepared in 10 volumes of buffer f (0.308 M tris-citrate) at pH 7.5 for studies with cystine, or buffer b (0.017 M tris) at pH 7.0 for studies with GSH, and incubated under an O_2 atmosphere. Further work will be necessary to determine what factor(s) resulted in the discrepancy between the results of Reynolds and Thompson (92) and the present experiments.

Reynolds and Thompson (92) also investigated the effects of a variety of other compounds on the inactivation of tyrosine aminotransferase. Analogues of L-cysteine, namely D-cysteine, cysteamine, β-mercaptoethanol, and β-mercaptopropionic acid, caused enzyme inactivation, but at a slower rate than L-cysteine. Certain analogues of tyrosine, namely dopamine, norepinephrine and epinephrine, also caused tyrosine aminotransferase inactivation at a slower rate than L-cysteine. L-Dihydroxyphenylalanine (L-DOPA), though, was found to be as effective as L-cysteine in causing enzyme inactivation, while tyrosine itself had no effect.

It was observed by Reynolds and Thompson (92) that of the compounds investigated those most similar in structure to one of the two substrates of tyrosine aminotransferase were the most effective in initiating enzyme inactivation. The substrate analogues may have been able to occupy the substrate sites of the molecule, and consequently have access to the pyridoxal 5'-phosphate bound to the enzyme. Reynolds and Thompson (92) suggested, therefore, that the compounds causing tyrosine aminotransferase inactivation reacted with pyridoxal 5'-phosphate removing the cofactor from the enzyme. The apoenzyme was then presumed to be susceptible to inactivation and degradation by an unknown mechanism.

The scheme proposed by Reynolds and Thompson (92) may be consistent with tyrosine aminotransferase inactivation by mixed disulfide formation. Reynolds and Thompson (92) observed that inactivation proceeded rapidly in the presence of cysteine or L-DOPA, but the loss of immunologically reactive material cross-reacting with antibody specific for tyrosine aminotransferase proceeded at a slower rate. These observations suggested that there was an intermediate inactivation step between removal of cofactor from the holoenzyme and further degradation of the apoenzyme (92). To be consistent with the present observations the inactivation step may represent mixed disulfide formation with the apoenzyme.

As described previously, tyrosine aminotransferase was less stable to heat denaturation (37) or proteolytic attack (4) in the apoenzyme form as compared to the holoenzyme. The same relationship may apply to inactivation by mixed disulfide formation. Reactive sulfhydryl groups found in or near the active site (42, 55) may be protected in the holoenzyme, but would likely be more susceptible to mixed disulfide formation in the apoenzyme.

Because significant levels of GSH or GSSG were expected to be present in the homogenates tested here, the possible role of these compounds in the inactivation of tyrosine aminotransferase has been considered in conjunction with the roles of cysteine, cystine and pyridoxal 5'-phosphate in the following paragraphs. GSSG has been assumed to be as effective as cystine in the inactivation of tyrosine aminotransferase under the conditions in the present experiments because GSH could substitute for cysteine in the inactivation of chicken liver tyrosine aminotransferase (section H of Discussion), and also because GSSG was found to cause the inactivation of several other enzymes (section D of Discussion).

In the present experiments tyrosine aminotransferase was only slowly inactivated, if at all, in homogenates incubated with no additions. Under an O_2 atmosphere endogenous GSH would likely have been oxidized to GSSG. Tyrosine aminotransferase apoenzyme was probably not readily formed under these conditions, and the concentration of GSSG may not have been sufficient to cause rapid inactivation of the holoenzyme.

Under N_2 atmosphere in homogenates containing cysteine, inactivation was inhibited for more than 3 h (Table 8). During this time tyrosine aminotransferase likely existed as the apoenzyme[1] as a result of complex formation between cysteine and pyridoxal 5'-phosphate. However, inactivation by the small amount of disulfides expected to be present in the homogenates was probably inhibited by the excess of cysteine (see sections D and F of Discussion). However, the excess cysteine may have been catabolized with the release of H_2S (see section C of Discussion), and if the catabolism of free cysteine did not disrupt the cysteine-pyridoxal 5'-phosphate complex, tyrosine aminotransferase may have remained in the apoenzyme form in the absence of free cysteine. The enzyme may then have been inactivated by the residual cystine or other disulfides present in the homogenate.

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[1] Full activity for tyrosine aminotransferase apoenzyme would be measured in the assay method used in the experiments reported here.

In the presence of added cystine, tyrosine aminotransferase inactivation was rapid and proceeded without a lag period (Table 12). Under these conditions the high concentration of cystine may have been able to compete effectively with pyridoxal 5'-phosphate for a site which would allow an exchange reaction with the essential sulfhydryl groups on the enzyme molecule.

In homogenates containing cysteine, complex formation between pyridoxal 5'-phosphate and cysteine may not have influenced the inactivation of tyrosine aminotransferase. Although a complex would likely be formed, the subsequent oxidation of cysteine would likely result in its dissociation (82). The cystine resulting from oxidation may have caused inactivation in the same manner as when cystine was added directly to the homogenate.

Litwack and Rosenfield (63) have recently emphasized that coenzyme dissociation may be the initial step in the in vivo degradation of enzymes. Similarly, Reynolds and Thompson (92) have proposed that the formation of tyrosine aminotransferase apoenzyme may initiate inactivation of the enzyme and its subsequent proteolytic degradation. The present results are consistent with this scheme and indicate that mixed disulfide formation may be the mechanism of inactivation subsequent to apoenzyme formation.

SUMMARY

With the aid of an automated technique for the assay of tyrosine aminotransferase activity, the inactivation of tyrosine aminotransferase in rat liver homogenates was studied under a variety of conditions. The measurement of inactivation was preceded by in vivo induction, and homogenization of the liver with a specially prepared loose fitting Potter Elvehjem tissue grinder. In a Krebs-Ringer-phosphate buffer with no additions inactivation proceeded slowly, whereas in a tris-citrate buffer with no additions the enzyme was stable. In either system the addition of both cysteine and O_2 caused a marked and significant increase in the rate of inactivation which was preceded by a lag period of about 1 h. It was found that if cystine were substituted for cysteine inactivation proceeded as before, but the requirement for O_2 and the duration of the lag period were significantly reduced. The particulate fraction of the rat liver homogenate was found to be required for inactivation in the presence of cysteine and O_2, but had little effect in the presence of cystine.

A search of the literature revealed that rat liver mitochondrial preparations are capable of catalyzing the oxidation of cysteine to cystine. Thus, it appeared that as a result of its oxidation cysteine may have caused the inactivation of tyrosine aminotransferase by the same mechanism as cystine.

This conclusion, however, does not agree with the function of cysteine proposed by Auricchio, Mollica and Liguori (3), who concluded that cysteine stimulated inactivation of tyrosine aminotransferase because of its activating effect on cathepsins B and B_1.

Auricchio, Mollica and Liguori (3), though, did not investigate the possibility that cysteine was oxidized in their homogenates. Nor does the present conclusion entirely agree with the effect of cysteine proposed by Reynolds and Thompson (92), who suggested that cysteine functioned by complexing with pyridoxal 5'-phosphate resulting in the formation of tyrosine aminotransferase apoenzyme.

Nevertheless, certain aspects of the three investigations are consistent with the model for tyrosine aminotransferase degradation proposed by Reynolds and Thompson (92). Auricchio, Mollica and Liguori (3) observed that tyrosine aminotransferase was inactivated by cathepsins B and B_1 prepared from rat liver lysosomes, which is consistent with the final step of the model; proteolytic degradation. The results of Reynolds and Thompson (92) indicated that the inactivation and degradation of tyrosine aminotransferase was preceded by formation of the apoenzyme, the first step of their model. The present results suggest that the second step of the model, inactivation, proceeds by mixed disulfide formation with the enzyme molecule.

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APPENDIX A

Tyrosine Aminotransferase Assay Techniques

References: Diamondstone, 1966 (17)

Granner and Tomkins, 1970 (30)

During the course of the experimentation several changes were made in the solutions used in the assay procedures. In one trial of experiment 1, one trial of experiment 2, experiment 6 and experiment 8 a phosphate buffer as described by Granner and Tomkins (30) was used. Although it is unlikely that the assay solutions could have had an effect on enzyme inactivation, phosphate was exchanged for tris in the rest of the experiments for reasons explained in the text.

Several changes were made in attempts to improve the operation of the automated technique. For example, Triton X-100 was found to prevent accumulation of solid material in the transmission tubing and flow cells. Separate experiments with the individual assay method showed that Triton X-100 had no effect on enzyme activity. The only other change which was effective was the splitting of solution F (used in the individual assays) into solutions B and G for the automated procedure. On standing at room temperature, solution F tended to form a white precipitate which made it unsuitable for the automated technique. Since none of the changes had any observable effect on tyrosine aminotransferase inactivation only the solutions finally used are presented here.

All solutions were prepared with distilled, demineralized water.

Solution A

0.20 M tris

1 mM EDTA

5 mg(ml)$^{-1}$ bovine albumin, fraction 5; added after the solution was made "up to the mark"

pH was adjusted to 7.88 at 25 C with concentrated HCl

Solution B

10.6 mM L-tyrosine (Sigma Chemical Co.)

5.32 mM sodium diethyldithiocarbamate (DDC) (Sigma Chemical Co.)

1.52 mM EDTA

0.05 % by volume Triton X-100

pH was adjusted to 10.5 with 10 N KOH

Solution C

0.277 M tris

2.92 mM DTT; added after pH adjustment

pH was adjusted to 7.5 at 25 C with concentrated HCl

Solution D

5.0 mM pyridoxal 5'-phosphate (Sigma Chemical Co.)

pH was adjusted to 6.5 with 1 N KOH

Solution E

0.50 M α-ketoglutaric acid (Sigma Chemical Co.)

pH was adjusted to 7.0 with 10 N KOH

In the preparation of solutions A to E the pH was adjusted before the solution was made "up to the mark". Solutions A to E were stored at 0 to 5 C and were used within two weeks after preparation.

Solution F

25 parts solution B
11 parts solution C
1 part solution D
1 part solution E

pH was adjusted to 7.88 at 25 C, if necessary, with concentrated HCl or 10 N KOH

Solution G

11 parts solution C
1 part solution D
1 part solution E

Solutions F and G were prepared immediately before use.

A1. Individual Tyrosine Aminotransferase Assay Method

1. 0.100 ml of homogenate was diluted to 1.00 ml with solution A.
2. 0.100 ml of the diluted homogenate was immediately added to 2.46 ml of solution F prewarmed to 37 C in a 25 ml erlynmeyer flask.
3. This mixture was incubated for 30 min at 37 C in a shaking water bath. The flasks were sealed with rubber stoppers during incubation.
4. At the end of the incubation 0.41 ml of 3.5 N KOH was rapidly added with shaking.

5. A second incubation for 30 min at 37 C followed.

6. The optical density was measured at a wavelength of 331 mμ[1] in a cell of path length 1.0 cm. A 10 × dilution with water was usually necessary.

7. Blanks were prepared by adding 0.41 ml of 3.5 N KOH to 2.46 ml of solution F immediately before the addition of diluted homogenate.

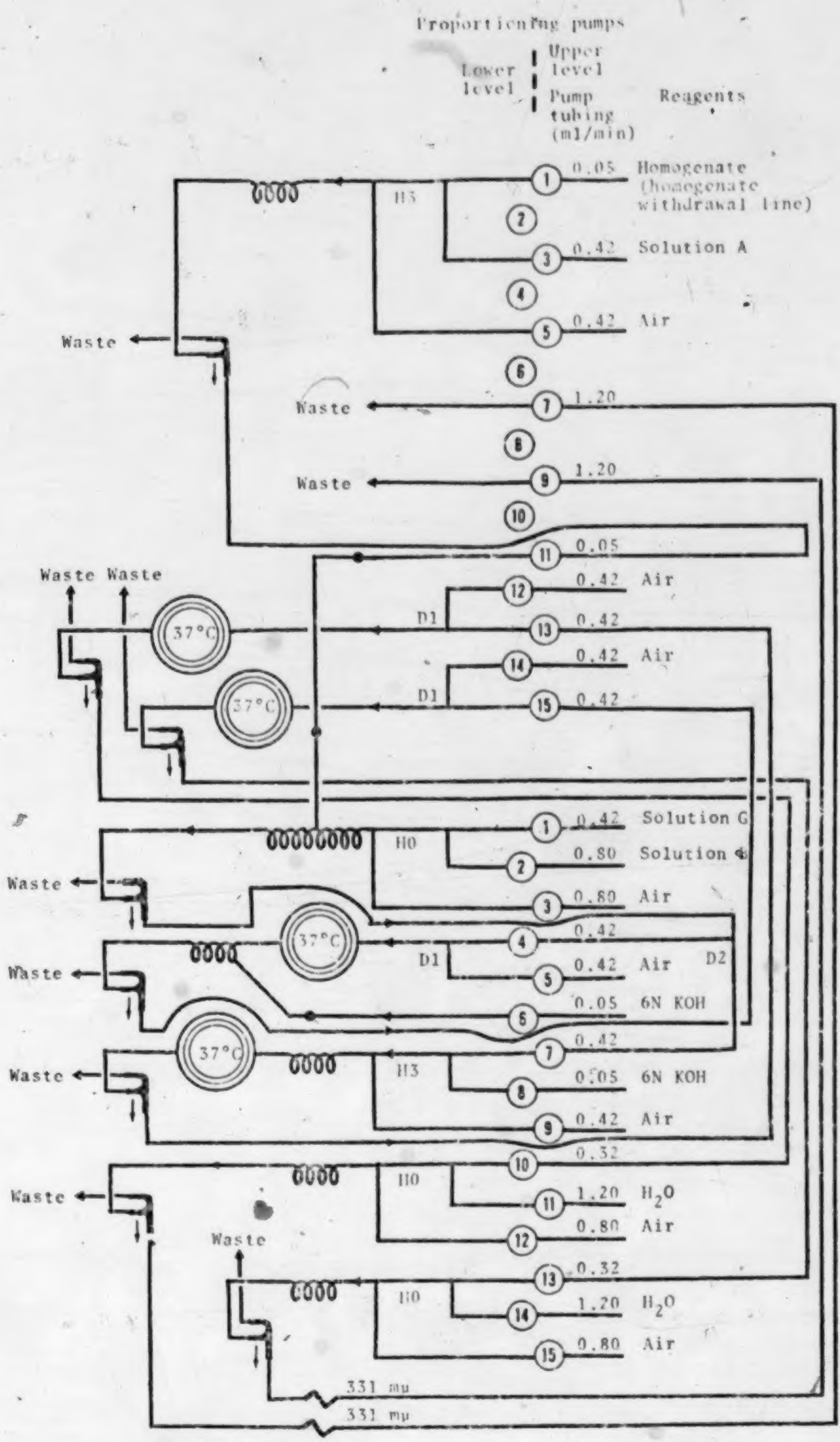
A2. Automated Tyrosine Aminotransferase Assay Method

The automated procedure was essentially the same as the individual assay method except that it was performed in a continuous manner. Homogenate was continuously withdrawn from the incubation vessel and diluted with solution A. Next, solutions B and G were added to the stream in the correct proportions. The stream was then split into two, a blank and a test. The blank received KOH immediately, whereas the test received KOH after the first incubation. Both incubations were carried out at 37 C in glass coils suspended in mineral oil baths. Both the test and the blank passed through two incubations, which were approximately 30 min each in duration. Following the second incubation, the two streams were passed through flow cells (1.0 cm path length) in a Gilford Model 2000 spectrophotometer (Gilford Instrument Laboratories) and the optical densities were recorded alternately (45 sec for each flow cell) on a

[1] A product of the transamination of tyrosine is *p*-hydroxyphenylpyruvic acid. The alkali catalyzed oxidation of *p*-hydroxyphenylpyruvic acid yields *p*-hydroxybenzoic acid, which absorbs maximally at 331 mμ.

Fig. 3. Flow diagram for tyrosine aminotransferase assay system. Auto Analyzer (Technicon Instruments Corp.) components were used unless otherwise noted. Code numbers (e.g., H3) refer to Auto Analyzer connectors. the water in the system (pump tubes 11 and 14, second proportioning pump) contained 0.05 % Brij 35 (30 % solution; Pierce Chemical Co.). Various components are represented by symbols:

——	Standard transmission tubing (1.59 mm ID).
—•—	Teflon tubing (0.38 mm ID) (Bel-Art Products).
ꝏꝏ	Single mixing coil (4 mm OD).
ꝏꝏ╲	Single mixing coil (4 mm OD) with capillary side arm. In this apparatus the side arm was fitted with sleeving to reduce the internal diameter.
ꝏꝏ┴ꝏꝏ	Double mixing coil (4 mm OD) with capillary side arm. In this apparatus the side arm was fitted with sleeving to reduce the internal diameter.
◎	Glass coil in heating bath. The coils were 12.2 m long, 1.6 mm ID and had a volume of 28 ml.
╗	Debubbler.
∿	Model 203 flow cell (1.0 cm path length) in model 2000 spectrophotometer (Gilford Instrument Laboratories).


Proportioning pumps
Lower level
Upper level
Pump tubing (ml/min)
Reagents
1 0.05 Homogenate (homogenate withdrawal line)
2
3 0.42 Solution A
4
5 0.42 Air
6
7 1.20
8
9 1.20
10
11 0.05
12 0.42 Air
13 0.42
14 0.42 Air
15 0.42
H3
Waste
Waste
Waste
Waste Waste
37°C
37°C
D1
D1
1 0.42 Solution G
2 0.80 Solution 4
3 0.80 Air
4 0.42
5 0.42 Air
6 0.05 6N KOH
7 0.42
8 0.05 6N KOH
9 0.42 Air
10 0.32
11 1.20 H_2O
12 0.80 Air
13 0.32
14 1.20 H_2O
15 0.80 Air
H0
D1
D2
H3
H0
H0
37°C
37°C
Waste
Waste
Waste
Waste
Waste
331 mμ
331 mμ

single chart. In most cases the automatic blank compensator attachment of the spectrophotometer was used so that the difference between the blank and test could be read directly from the chart. See Fig. 3 for a detailed description of the Auto Analyzer (Technicon Instruments Corp.) equipment used in the automated procedure.